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TO MY PARENTS AND MY DAUGHTERS

KHLOD and SARA

ASPECTS OF THE GROWTH AND DEVELOPMENT OF THE  
SPORANGIOPHORE OF PHYCOMYCES BLAKESLEEANUS

by

HAMOUD ABDOU SAADI

B.Sc. (Riyadh, Saudi Arabia)



A Thesis submitted to the University of  
Durham for a Degree of Doctor Philosophy

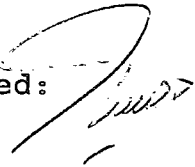
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DECLARATION

The work described in this thesis has been carried out by the undersigned at the Department of Botany of Durham University and has not been previously submitted for any other diploma or degree.

Signed: 

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ABSTRACT

Chapter I of this study deals with the distribution of cellular components and growth during the differentiation and morphogenesis of the reproductive structure, sporangiophore, of Phycomyces blakesleeanus. A non-uniform distribution of nuclei, mitochondria, nucleic acids, protein and cell wall components throughout the sporangiophore of stage I was found. The above results gave an understanding of (i) the organization of the cytoplasm in the growing region of the sporangiophore (with especial emphasis on the apical region); (ii) the possible importance of a balance between polymer synthesis and hydrolysis in growth regions; (iii) the probable location of wall synthesizing and lysing enzymes.

In Chapter II of this investigation, the isolation and development of protoplasts is described. A different capacity for wall production and development from protoplasts released from different region of sporangiophore is described. Protoplasts from the tip region were found to have a great concentration of nucleic acids and protein and also a greater activity in wall production. A correlation between the regeneration of protoplasts and the integrity of the membrane in wall morphogenesis in general is described.



In Chapter III, the biochemical observation of the distribution and recovery of RNA species and ribosomes during growth and development of the sporangiophore is presented. Isolations and characterizations are described. The non-uniform distribution of macromolecules was found to be associated with greater recovery of rRNA and ribosomal polymers. The maintenance of the apical growth was seen to be associated with the location of greater quantities of rRNA, ribosomes and also appeared to be dependent on the existence of preformed mRNA. Abnormal production of wall substances was found to be associated with the breakdown of the rRNA and ribosomes fractions.

Conclusions reached from this work are that the sub-apical region of the sporangiophore contains the necessary components to maintain a high synthetic capacity for proteins and cell wall precursors. This non-uniform distribution of metabolism appeared to be maintained at the expense of other regions; it may well also provide the basis for the polar properties and the general behaviour summed up by the term 'apical dominance'.

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### List of Abbreviations

The following abbreviations are used in the text and where appropriate, to label all Plates presented in this thesis:-

A - Apex

C - Columella

DNA - Deoxyribonucleic acid

DNAase - Deoxyribonuclease

DOC - Sodium deoxycholate

E - Extinction at a designated wavelength

E.coli - Escherichia coli

ER - Endoplasmic reticulum

EX - Extension zone

EDTA - Ethylene diamine tetra acetic acid (disodium salt)

FC - Foot cell

GZ - Growing zone

Mi - Microfibril

M - Mitochondria

N - Nuclei

O.D. - Optical density

PCA - Perchloro acetic acid

PAGE - Polyacrylamide gel electrophoresis

RNA - Ribonucleic acid

RNAase - Ribonuclease



mRNA - Messenger RNA

Mol. wt. - Molecular weight

MS - Mega spore

rRNA - Ribosomal RNA

tRNA - Transfer RNA

S - Svedberg value (sedimentation coefficient)

SDS - Sodium dodecyl sulphate

Spph - Sporangiphore

Sp - Sporangium

TEMED -  $\text{N}:\text{N}:\text{N}:\text{N}^1$  - tetra-methyl-1:2-diamino ethane

TCA - Trichloroacetic acid

u.v. - Ultra violet

## 1. GENERAL INTRODUCTION

Phycomyces blakesleeenanus Burgeff is a true fungus, a lower eukaryotic saprophytic organism. It belongs to the class Zygomycetes, order Mucorales. The genus Phycomyces which was discovered by Kunze (1823), belongs to a sub-family whose multinucleate vegetative spores have been characterized.

The life cycle and requirements for development of this particular fungus have been extensively reviewed by Bergman et al., (1969), Fincham et al., (1979). The ungerminated vegetative spores, were characterized as being ellipsoidal, non-motile, multi-nucleate, and surrounded by a thick wall. Before germination the spores show low metabolic activity (see Gregory, 1966) which permits survival for long periods until environmental conditions become suitable for germination. Germination of spores is followed by subsequent development of coenocytic mycelium. The asexual life cycle of this fungus is initiated with the formation of foot cells on the substrate hyphae from which aerial hyphae (sporangiophores) develop. During growth and development of the sporangiophore, four stages of different morphogenetical development have been recognized and classified by Castle (1942) as

stages I, II, III and IV a & b. The stage I sporangiophore has been characterized as having a pointed tapered tip (Trinci and Halford 1975). Subsequent morphogenesis was shown to be achieved by swelling and maturation of the tapered tip region forming the so called 'sporangium'. Further investigations of growth differentiation and morphogenesis of sporangiophores are described by Castle (1958, 1959), Goodell (1971), Cochrane (1974), Burnett (1976), Gamow and Bottger (1977, 1979), Cerda-Olmedo (1977) and Galland & Russo (1979).

Numerous studies have been also made on the behavioural responses of the different stages of sporangiophore growth to such factors as light, temperature, avoidance and gravity (Bergman et al., 1969; Bergman, 1972; Johnson & Gamow, 1971, 1972; Dennison and Bozof, 1973; Ortega et al., 1974; Cerda-Olmedo, 1974; Dennison & Foster, 1977). Recently it was suggested that the sporangiophore may be considered as an olfactory organ (Cohen et al., 1979).

Apical growth in the green plant has been an attractive subject for many biologists (Wilkins, 1969; Roberts, 1976). It has been shown that the stem growth of the plant is controlled by special differentiated cells which have been located at the apex region of the stem. Alternatively, the fungus Phycomyces has been chosen as

a model eukaryotic organism for manipulation and study of the apical growth and for investigation into the effects of the apical region on the rest of the parts of the sporangiophore (see Gamow & Goodell 1969; Goodell 1971). Gruen (1959, 1967), Gruen & Ootaki (1970, 1972), and Ootaki & Gruen (1970) found that the isolated sporangiophore could grow and undergo the same sequence of morphogenesis as if still attached. The amount of cytoplasm and its redistribution or reorganization within the sporangiophore has been suggested as an important factor in controlling this growth since the migration of the cytoplasm supplied from the substrate-hyphae through the sporangiophore was found to cease at the initiation of stage II (Bergman et al., 1969). Degradation of older organelles in the older region along the sporangiophore and utilization of the degraded substances in the younger regions has been used as an explanation of the dominance of the tip region.

The fact that fungal hyphae and sporangiophores grow apically has also been established by Burgeff (1915), Castle (1958, 1959), Bergman et al., (1969), Burnett (1976), Trinci (1978b). However, it has been conclusively established, by autoradiographic labelling of hyphae of different fungi, with cell wall constituents, that

microfibril constituents are preferentially deposited in the hyphal apex of fungi and that maximum incorporation occurs at the extreme apex, by Bartnicki-Garcia & Lippman (1969), Katz & Rosenberger (1970, 1971), Gooday (1971), Collinge & Trinci (1974) and reviewed by Grove (1978), Gooday (1978) & Trinci (1978b). Further, the integrity of the sporangiophore has been shown to be regulated by the tip region (1-2 mm, Trinci & Halford, 1975; Gruen & Ootaki, 1972). It was suggested (Trinci, 1978b) that the growth extension at the site where the new wall is deposited can be maintained by a balance between the wall lysis at the extreme apex and wall deposition at a region at the basal end of the tapered tip region. Additionally, vesicles and other membranous like structures have been found to be important organelles in synthesizing and carrying the wall precursors of fungi (Weber & Hess, 1976). The accumulation of the vesicles and membranes at the apical region of the sporangiophore (Peat & Banbury, 1967; Marchant et al., 1967; Thornton, 1968b; Bergman et al., 1969; Hankinson, 1972; Tu & Malhotra, 1973, 1976; Trinci, 1978b), was suggested to be associated with extension activity and with new wall synthesis; also it was reported that vesicle formation and migration might be under the control of the sub-apical region. Vesicles thus may migrate to

the apex where they may eventually coalesce with the apical plasmalemma (Trinci, 1978b). Thus, the vesicles may accomplish two essential functions: they may extend the plasmalemma surface and also discharge enzymes (Girbardt, 1969 and Bartnicki-Garcia, 1973). This is only a suggestion, since the exact function of vesicles is as yet not known. However, the idea that vesicles could possibly carry the wall precursors of the filamentous fungi was recently reviewed by Rosenberger (1976), Stewart & Rogers (1978).

The apical extension of the Phycomyces hypha has been shown to be maintained by a sharp gradient of active chitin synthase that is rapidly inactivated during the transition from tip to the lateral wall (Ruiz-Herrera & Bartnicki-Garcia, 1976b). Additionally particulate zymogen, chitin synthase, was also isolated and tested by Thomson & Fischer (1976) and it has been found to be activated by protease (see also Fischer & Thomson, 1979). The presence of considerable enzyme activity in the sub-apical region of the sporangiophore in addition to the presence of more ribosome, also gives an indication of high metabolic activity of the tip region in synthesizing protein and building new wall (see Jan, 1974; and Gooday, 1978). The relationship between apical extension

and protein synthesis was shown by the effect of cycloheximide in inhibiting the apical growth of hyphae of Aspergillus nidulans (Katz & Rosenberger, 1971) and growth of Schizophyllum commune (Vries & Wessel, 1975). Inhibition of spore germination in Phycomyces by cycloheximide and 5-fluorouracil analogue was also reported by Van Laere et al., (1976, 1977).

Thus RNA and protein synthesis appeared to have an important function in controlling cell wall formation and thus the growth of the hyphae (see review by Grove, 1978; Ojha & Dutta, 1978). The inhibition of spore germination of Phycomyces was attributed to an inhibition of chitin synthesis at the translation level (Van Laere et al., 1976, 1977).

No further investigations have been reported to illustrate the effect of protein and RNA metabolism on the cell wall formation and growth extension of the sporangiophore of Phycomyces. Such a study is important since it has been reported that the change in the properties of macromolecules are reflected in the whole organism and represent one of the principal causes of natural ageing of living matter during spontaneous degradation in time (Fencel, 1978). Likewise cells resynthesize sub-cellular units but some of the change,

e.g. in nuclear material and in cell walls, are irreversible and hence bear directly on the viability of the cell. During the starvation or imbalanced condition some cytoplasmic components, particularly RNA, have been found to be degraded and utilized as biosynthetic precursors for synthesizing cell material (see Hespell & Mertens, 1978). Maintenance of apical tip growth during starvation of some fungi (see Trinci & Righelato, 1970; Gooday, 1978; Fencel, 1978) has been found to occur at the expense of other parts of the hypha. Additionally the tip region of fungal hyphae has been shown not to be exposed to starvation as are older parts of the hyphae and therefore it retains a higher level of RNA and protein and thus regeneration ability (Macheck & Fencel, 1973). Growth of sporangiophores during isolation or during stage IV can be considered to be under starvation conditions where the apical growth extension is still maintained. Unique distribution of organelles and components at that region may also be contributed to its resistance and viability.

Any further investigation for the reorganization and determination of the redistribution of the cytoplasmic components and organelles during the regeneration of the sporangiophore should achieve a better understanding of



the apical dominance and thus control of sporangiophore growth (see Goodell, 1971; Gruen & Ootaki, 1972). This study aimed firstly to investigate, by cytological observations, the role of the cytoplasmic organelles in the growth and development of the sporangiophore, especially of stage I. Secondly the involvement of the protoplasts in extension and regeneration. Thirdly the distribution of RNA and ribosomes during sporangiophore growth.

It was envisaged that the findings of this study would contribute to the knowledge of the growth and development of Phycomyces.

## 2. GENERAL MATERIALS AND METHODS

Phycomyces blakesleeana Burgeff CBS Baarn (-) strain was used in the present work. It was grown on 2% malt-agar medium in petri dishes.

### 1. Growth condition

#### 1.1 Sub-Culture methods

Sub-cultures were maintained by one of the following processes, keeping the culture in a healthy condition. Firstly it was routinely maintained by transferring the inoculum on an agar block (1 cm<sup>2</sup>) carrying mature mycelium already grown, and producing sporangiophores. The blocks of inoculum were placed on the surface of the new medium. Secondly, an inoculum from a solution which contained spores (after harvesting and activating) was also employed in maintaining stock cultures. Spores from the culture supplied were harvested from a dense crop of mature sporangiophores (stage IV). The surface of the culture was wetted with autoclaved sterilized water (5 ml for a single plastic petri dish). This was followed by shaking the petri dish). Alternatively, mature sporangiophores were picked up with fine sterilized forceps. The contents of a sporangium were squeezed out and mixed with sterile water in a small flask.

The spore suspension thus produced, was then transferred to the sterilized water flask of 50 ml capacity.

Before inoculating, spores in suspension were activated at  $48^{\circ}\text{C}$  for 8 minutes. Heat activation was associated with shaking. 1 ml of spore suspension was usually used to inoculate 350 ml of liquid medium in 2L capacity flask or onto solid medium in a petri dish. Heat shock was used to break the dormancy of the spore and activate the germination (see Ramadani, 1978). To obtain mycelium, inoculated liquid medium was shaken on an orbital shaking machine (150 rev/min. ) to agitate the spore suspension from inoculation, while mycelial development continued. After 48 hours of shaking, flasks containing already grown mycelium were transferred to a growth chamber to produce sporangiophores. Growth was continued under overhead illumination, from 20 W fluorescent light and temperature of  $23 \pm 2^{\circ}\text{C}$ , see Plate 1(1). Keeping the flasks with the grown mycelium in a shaking condition, showed to inhibit the growth of sporangiophore. The capability of the mycelium to produce a floating aerial substrate-hyphae and then produce a sporangiophore was apparent when the culture was kept without shaking. Inoculated petri dishes were also kept in the growth chamber under similar conditions as described above.

## 1.2 Growth media

Ødegar's (1952) minimal medium was usually used for producing a maximal growth of Phycomyces. The following contents were dissolved in 1L water:

|   |         |
|---|---------|
| D-Glucose                                 | 30 gm   |
| L-Asparagine                              | 3.5 gm  |
| $\text{KH}_2\text{PO}_4$                  | 1 gm    |
| $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ | 0.5 gm  |
| $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ | 80 gm   |
| $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ | 0.44 mg |
| $\text{FeCl}_2 \cdot 6\text{H}_2\text{O}$ | 0.32 mg |
| Thiamine                                  | 50 mg   |

The chemicals were of Analar grade and the water was glass distilled, but no special precautions were taken to exclude minute traces of other substances in the water or chemicals employed.

Solid medium was achieved by addition of  $20 \text{ gm} \cdot \text{L}^{-1}$  agar to the previous medium. Prepared medium and containers were autoclaved for 15 minutes at  $120^\circ\text{C}$  under  $1 \text{ Kg} \cdot \text{cm}^{-3}$ .

Nitrogen limited medium was achieved by addition of  $1.5 \text{ gm} \cdot \text{L}^{-1}$  Asparagine to the previous medium instead of  $3.5 \text{ gm} \cdot \text{L}^{-1}$ . This was used in some cases during the induction of synchronization (see Smith, 1978) and regeneration of sporangiophores.

### 3. METHODS FOR SPORANGIOPHORE SYNCHRONIZATION

Four stages of Phycomyces blakesleeianus sporangio-  
phore growth and development were observed arising  
from the surface of inoculated media. Stage I sporangio-  
phores were the main object of the present study. To  
provide material for precise analysis, it became  
desirable to achieve nearly synchronized growth and  
development. Two main procedures have been used.

#### Treatments to achieve synchronization in development

##### 1. From a spore suspension

A spore suspension was prepared as mentioned above.  
The spores were centrifuged and the spore pellet washed  
repeatedly with sterilized distilled water. A volume of  
20 ml was used in each step, accompanied by vigorous  
shaking to break up any spore clumps, which might give  
irregular spore germination and less synchronization in  
growth. Activation of the washed spores, free from  
clumps, was brought about by repeated heat-shock for  
2-3 minutes at a temperature of 30°C in a water bath.  
Each shock was a treatment which was repeated three to  
five times, with cooling between each treatment. This  
was found to produce a good yield of spores showing  
synchronization of germination. Increasing the  
temperature and the time of heat activation impaired  
spore viability and synchronization of germination.

Inoculation with washed activated spore suspensions produced in this way, can be used for both liquid and solid media, and it resulted in synchronization in the growth and developmental stages of sporangiophores.

Another method was sometimes employed which was a modification of the procedure described above. Young mycelium which was growing in shaken liquid culture was filtered off by suction onto filter paper or filter cloth. The filtrate was washed with sterilized distilled water. The mycelial mat produced in this way was cultivated on a shallow layer of liquid medium. The shallow layer of the liquid medium was covered with a permeable membrane (cellophane), keeping the mycelium floating over the surface of the liquid medium. The permeability of the wetted membrane was not tested; it was well wetted with the liquid medium and provided effective support for surface growth. The implication of the method is to induce the formation of the sporangiophore by a sudden aeration of the mycelium. The sudden aeration of the vegetative cultures of filamentous fungi has been shown to give synchronization (Smith, 1978). This method gave a good synchronization of sporangiophore production. The practice of using a shallow layer of liquid medium was shown to produce a small yield of sporangiophores. However, using a large quantity of

liquid medium such as 350 ml, has been shown to produce dispersed and loose mycelia.

2. From a substrate-hyphae already grown on agar minimal medium

A piece of agar (equal to a whole area of a solid medium in the petri dish) carrying the substrate-hyphae, was placed gently onto the surface of the liquid medium (350 ml in 2L flask). Care was taken to avoid the sinking of the agar pieces in the liquid medium. The substrate-hyphae which were grown on the surface of the agar developed better, from spores which had been treated to activate germination and also growth synchrony (see above). Glass casserole dishes were also used as growth vessels in large scale production of sporangiophore, bearing in mind that all growth vessels and medium were autoclaved. A good yield of synchronization and growth were obtained by this method.

CHAPTER I

Growth and Cytological Studies



#### 4. INTRODUCTION

Cytologists have long been searching the hyphal tip of fungi for internal structures that might offer a clue to the mechanism of apical growth. Brunswick (1924) and Girbardt (1956) found a plausible candidate for the cytological basis of apical growth in a densely staining, apical body, 'spitzenkörper' seen in the tips of fixed hyphae of Coprinus. Moreover Girbardt (1957) McClure et al., (1968) and reviewed by Bartnicki-Garcia (1973), and Grove (1978) found the changes in the direction of hyphal growth could be correlated with prior changes in the position of the 'spitzenkörper' at the tip of the hyphae of the Ascomycetes and some Basidiomycetes.

The different structures of the tip region of filamentous fungi were extensively reviewed by Burnett (1976). Early indications were that the protoplasm of the growing tip of the hyphae of Phycomyces were structurally and functionally different from that of the rest of mycelium in that they had an apical cap like structure (Bergman et al., 1969; Burnett, 1976) as do other related members of the Zygomycetes (McClure et al., 1968; Girbardt, 1969; Grove & Bracker, 1970). Such structures were defined

as vesicles, see reviews by Grove (1978) and Trinci (1978b).

Investigations into the internal structure of the sporangiophore during growth and development were carried out by Marchant et al., (1967), Peat & Banbury (1967), Thornton (1968a,b), Zalokar, (1969), Bergman et al., (1969), Flanagan (1970), Tu et al., (1971), Frank & Reau (1971), Hankinson (1972), Tu & Malhotra (1973, 1975, 1977), using both electron and phase contrast microscopy. It has been shown that the growth and development of the sporangiophore was associated with the differentiation of cytoplasmic organelles. Different shapes of the mitochondria have been found along the axis of sporangiophore (Tu & Malhotra, 1975; Tu et al., 1971), which suggests the presence of differences in metabolic activity. The non-uniform distribution of the cellular components was found associated with apical extension of the tip region of the sporangiophore. Use of the electron microscope showed that the apex region had a greater concentration of vesicles, with the apparent lack of other organelles.

Nuclei, ribosomes, mitochondria and endoplasmic reticulum were found most abundant in the sub-apical region. An understanding of the growth of the highly

polarized region of the sporangiophore tip, is necessary to illustrate the inhibition or stimulation of growth (Ootaki & Gruen, 1970). Further investigations are needed into the interrelationships between induced differentiation and morphogenesis and the distribution of organelles along the sporangiophore. The correlation between the distribution of the RNA, nuclei and protein during wall synthesis could be an important factor in determining wall synthesis activity during regeneration and growth (Gamow & Goodell, 1969; Goodell, 1971; Gruen & Ootaki, 1970, 1972).

The gradient in the activity of the chitin synthase at parts distant from the tip region (McMurrough & Bartnicki-Garcia, 1973; Ruiz-Herrera & Bartnicki-Garcia, 1976b) could possibly be related to the gradient activity of organelles as suggested in fungi in general by Lahoz et al., (1970), Fencel (1978). The accumulation of RNA at the tip of Neurospora crassa was shown to be associated with a decline in concentration of RNA at the parts below the tip region (Zalokar, 1959b). An increased supply of liberated compounds possibly are needed for maintaining apical growth of filamentous fungal hyphae (Fencel, 1978; Martin & Demain, 1978; Stewart & Rogers, 1978).

The effect of the organelles such as ribosomes and mitochondria in controlling the differentiation and morphogenesis of fungi, have been shown during growth of zoospores of Blastocladiella emersonii and Allomyces and conidia of Neurospora crassa (Cantino et al., 1963; Turian & Viswanathan, 1966; Turian, 1975; Lovett, 1975). The growth of these above fungi was shown to be correlated with the free nature of ribosomes and mitochondria in the cytoplasm. The activity of the sporangiophore of Phycomyces has been shown to be affected by transplanting of nuclei (Ootaki et al., 1974, 1977). This proved that nuclei survived and maintained their functional activity during the manipulation. Correlations between the nucleic acid and protein, during growth and differentiation of the hyphae of filamentous fungi have been recently reviewed by Ojha & Dutta (1978), Wright (1978), Bramble et al., (1978), Bull & Trinci (1977), Smith (1978), Martin & Demain (1978), Walsh & Wright (1978). It is known that hyphal tip growth is the principal means by which filamentous fungi expand their hyphae. Hyphal tip growth has been extensively described by Bergman et al., (1969); Grove (1978), Trinci (1978b), although the precise mechanism of incorporation and regulation of material for hyphal extension has yet to be fully explained (Burnett, 1976). There is, however, a

considerable amount of ultrastructural and biochemical evidence to suggest that the apex differs both structurally and cytochemically from the main body of the hyphae (see Zalokar, 1959a,b; Gull & Trinci, 1974; Steel & Trinci, 1975; Burnett, 1976). For example, protein, RNA, nuclei and mitochondria are distributed fairly evenly throughout actively growing undifferentiated mycelia or leading hyphae (Nishi et al., 1968; Fencel et al., 1969; and see review by Trinci, 1978a,b). Trinci (1978a) cited that some morphological and biochemical gradients occurring in hyphae and in particular, the tips of hyphae, are always morphologically and biochemically differentiated from other parts of the mycelium, e.g. they contain vesicles (Grove & Bracker, 1970) and are rich in chitin synthase activity (Gooday, 1971) but lack nuclei and mitochondria (Zalokar, 1959b). Trinci (1978a) also suggested that, mechanisms which relate and integrate the synthesis of cellular components during growth and development of fungi exist in the more or less homogenous distribution of macromolecules, enzymes and organelles in the apical compartments of leading hyphae. Since it is necessary to know which constituents of the cellular components are involved and more particularly which of these are critical and limiting to the rate of the wall extension of sporangiophore, the location of

nuclei, mitochondria and ribosomes is of importance as suggested by work in living systems by McClure et al., (1968), and reviewed by D'amato (1976), Nagl (1976), Novikoff & Holtzman (1976). Information from the light microscope with selective staining methods and physiological studies should all add to the current understanding of the cytology of hyphal tip growth of filamentous fungi. It is now widely accepted that the primary critical factor(s) controlling cellular differentiation is differential gene activation (Axelrod et al., 1973; Lewis, 1974, 1976; Orlowski & Sypherd, 1977, 1978a,b; Smith, 1978). Thus qualitative and/or quantitative changes in rates of nuclei, nucleic acids and protein synthesis are believed to be major variables limiting differentiation.

## 5. MATERIALS AND METHODS

### 1. Growth and measurements

#### 1.1 Measurement of growth of attached sporangiophores with complete tip

##### 1.1.1 The length

Length measurement was commenced at the time of initiation of the pointed tip of the sporangiophore. A calibrated travelling microscope was used. The culture was placed on the level of the optical axis of the microscope. Care was taken to minimize handling of the cultures or any manipulation, which might lead to abnormal results. Under suitable conditions, growth was measured at intervals of 0, 2, 4, 8, 12, 24, 48 hrs. The length measurements were plotted against the age of the sporangiophores. This also could be done on the second crop of sporangiophores (Gruen, 1959). Obtaining second or several consecutive crops was achieved by harvesting the previous crop of sporangiophores using fine sterilized forceps, and taking care to avoid damaging the surface mycelium.

##### 1.1.2 The weight

Dry weights were routinely used for growth measurements. They were made by harvesting sporangiophores at the same periods of measuring length, as shown above. Similar culture was also used. Dry weight was obtained by placing the sporangiophores

in the oven at 50°C until constant weight values were obtained. Each reading was repeated three times, the mean values were calculated and were plotted against the length of sporangiophores.

1.2 Measurement of growth of isolated sporangiophores (intact structure)

1.2.1 Isolation and subsequent culture in liquid medium

The culture chamber for isolated sporangiophores was set up in the manner illustrated in Plate 1(2). Sterile transparent cylindrical jars, 9 cm high, with screw lids about 9 cm in diameter (A), served as an outer enclosure. When the jars were inverted, an open petri dish culture could be fitted into the lid, and it was covered by the transparent cylindrical body of the jar. A small amount of petroleum jelly was smeared along both long edges of glass slides.

Liquid nutrient medium or water was added to the small inner dish (B) to a depth of about 0.7 cm. Addition of liquid solution to a greater depth than 0.8 cm was liable to wash loose the isolated sporangiophores.

Controls with sterilized water were also included. Sporangiophores were plucked up from the mycelium with very fine forceps and gently attached to the edges smeared



with petroleum jelly. Seven to eight sporangiophores could be stuck along one side, and the same number along the second side of the long edges.

The slide was placed as in B across a 5 cm diameter dish with the basal ends of the sporangiophores dipping into liquid medium or water, and the assembly centrally placed with the small dish centrally placed in the lid of the inverted jar, which when replaced, covered and protected it. The minimal medium was tested for freedom from contamination by streaking a loopful onto the solid medium (see above) incubating and examining for colonies of any contamination. Each jar was incubated in the experimental growth chamber (photographed in Plate 1(2)) with overhead fluorescent light (20 W) and at room temperature  $23 \pm 2^{\circ}\text{C}$ . Synchronized sporangiophores in stage I of different initial lengths 10, 15, 20, 25, 30, 40 mm were used. Each length experiment was repeated three times, taking into account that all of the sporangiophores were nearly at the same age and same length. Increases in length were measured as explained above at intervals of 0, 2, 4, 8, 12, 24, and 48 hours, and the dry weight was determined at 0, 12 and 24 hours.

As an alternative to sticking the sporangiophores against a petroleum smeared edge of a slide, sporangiophores

were also placed inside the open top of a test tube nearly filled with liquid medium or sterilized water. The base of each sporangiophore dipped into the liquid and a film held by capillary action between the glass and the lowest few millimeters of the sporangiophore served to retain them in position as shown in Figure (1(1)).

#### 1.2.2 Isolation and subsequent culture in solid medium

Isolated sporangiophores of stage I were also cultured on the solid minimal medium (containing 20 g.L<sup>-1</sup> agar). The method has been modified from that used by Gruen & Ootaki (1970, 1972). Agar blocks were cut into pieces 5 mm in length 2 mm in width and about 3 mm deep. This size was the same for all experiments. Sporangiophores 10-20 mm in length were carefully placed on the agar piece. The tapered apex and open base of each sporangiophore overlapped the agar and extended beyond it (see the diagram in Figure (1(2))). Great care was taken to avoid damage to the sporangiophore and particularly to its apex. For control and comparison, isolated sporangiophores were also cultured in water.

The capacity of the isolated and manipulated sporangiophores to grow and regenerate was measured at intervals corresponding to the usual length increase measurement.

All cultures in protective jars, were incubated in the illuminated chamber, under the usual conditions.

1.3 Measurement of growth of isolated sporangiophores (in segments)

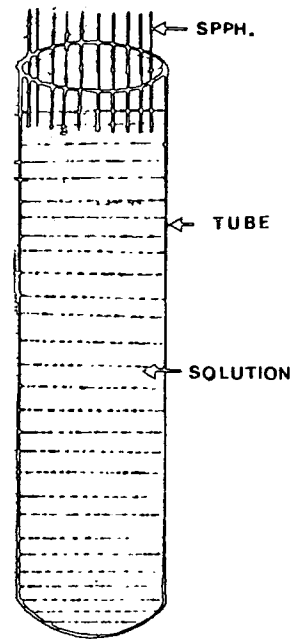
1.3.1 Isolation and subsequent culture of sporangiophore segments

Using the same incubation procedure as for full length sporangiophores, experiments were also undertaken with sporangiophores cut into segments. The cutting up, was undertaken, using sterilized fine scissors and forceps, or stainless steel razors with platinum hardened edges. Before the cutting, each sporangiophore was placed on a 2% agar block as a supporter, then a sharp perpendicular cut was achieved without squashing the sporangiophore edges. Sporangiophores were cut into approximately equal segments of 3-5 mm starting with a certain stage I sporangiophore about 15-20 mm long. The extreme apex of the sporangiophore or the tapered tip (1-2 mm) was included as part of the apical segment in some instances, and cut away from the apical segment in others. Figure 1(3) shows diagram of the segments along sporangiophore of stage I.

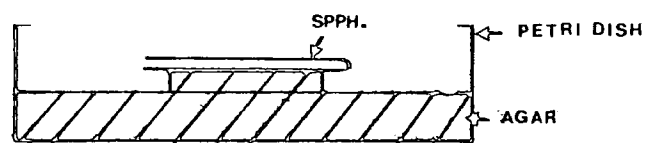
Segmented sporangiophores were cultured on solid minimal medium, observing the procedures and precautions mentioned above, and also on water as a control.

Figure (1)

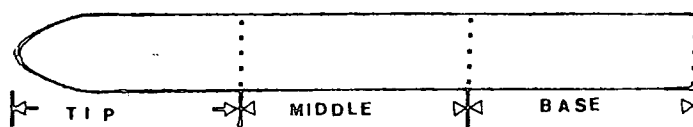
1. Diagram showing the system for culturing isolated sporangiophores inside the open top of a test tube.
2. Diagram showing sporangiophore during culturing on a piece of solid medium.
3. Diagram of segments, cut from sporangiophore of stage I.



1



2



◀ SUB-APEX ▶

3

Analysis procedures were followed as mentioned above for length increase and weights measurements, under similar growth conditions. For each segment, observations were made of the number, nature and location of regenerated structures at each end. Corresponding records were also kept for sporangiophores that were not subdivided into component segments. The experiments were repeated three or four times to determine the consistency of the results.

## 2. Cytological study

The cytological experiments covered studies of the processes of:-

### 2.1 Fixation

### 2.2 Washing and mounting

### 2.3 Staining of nuclei

Small cubic glass wells (3 cm x 3 cm x 1 cm) provided with tight fitting stoppers were used for the process. Glass jars with fitted stoppers were also used. The later ones were used when a complete slide carrying the preparation needed to be immersed in the solutions, while the former equipment was used when individual sporangiophores were manipulated.

### 2.1 Fixation

As a preliminary test, freezing with solid CO<sub>2</sub> and drying with absolute ethylalcohol, was used with the aim

of obtaining a good fixation, but this gave poorer preparations. Fixation was also carried out under vacuum, in order to facilitate the penetration of fixative solutes and also in obtaining uniform time fixation. This method was found to take longer periods and at times cytoplasmic dislocation and disorganization was seen.

Many trials were made, aimed at achieving proper fixation of sporangiophore contents with the natural location of protoplasmic constituents preserved. Sporangiophores were carefully plucked up from the cultures, so no internal damage was caused. Different kinds of fixatives were used: 2-4% glutaraldehyde in 0.1M phosphate buffer at pH 7.0, acetic acid-alcohol (1:3 v/v, Carnoy fixative), formalin-acetic acid-alcohol (2:1:3 v:v:v), formalin-acetic acid (2:1 v/v), formalin-alcohol (2:3 v/v), and 1% solution osmium tetroxide, were used (see Peacock, 1958; Gurr 1960; Jensen, 1962).

Different ways of manipulating sporangiophores during fixation were also employed. Sporangiophores were fixed in situ (at the time when sporangiophores were attached to the parent culture). A piece of agar carrying surface mycelium and sporangiophores or a piece

of the substrate-hyphae with sporangiophores sparsely distributed, were cut out of the culture and submerged in one of the fixatives. This method was used to minimize the effect of the mechanical handling, which might produce preparations with displacement and disorganization of sporangiophore contents. The isolation of sporangiophores, with very fine forceps, was shown to give no dislocation or disorganization. Time and resolutions of the preparations are shown in the staining method.

Sporangiophore cuticles present difficulties in penetration of the solutes of fixatives, and during washing and staining. Attempts were carried out to minimize its effect. Treatment of sporangiophores with 0.2% of each of Tween 80 and Triton X-100 for 15 minutes before the fixation, was employed. Sporangiophores were also fixed with fixative solutions which contained 0.2% of each of Tween 80 and Triton X-100. Comparing these two methods, the latter method gave a better penetration of fixative's solute. The disadvantage of this method was the bursting of the tip region of sporangiophores which were shown in some preparations.

Treatment of sporangiophores with 0.1M KOH (Carnoy, 1870) for 13 minutes before the fixation, has been shown



to give better observations than those described above. Most of the other fixatives did not give as good resolution and uniform fixation as did acetic acid-alcohol. In addition acetic acid-alcohol fixative also gave a good staining for RNA, DNA, nuclei and protein, since it was considered (Jensen, 1962) to be the best fixative for the macromolecules. The sporangiophores were also treated with 0.1M KOH, then washed in distilled water for 2-3 minutes to remove any excess of the base and then fixed in acetic-acid alcohol. After fixation, washing the sporangiophores free from acid and mounting the specimen were necessary steps in the cytological study.

## 2.2 Washing and mounting

Fixation procedures usually should be followed by washing away any excess of the fixative to prevent interference with the staining solution and to minimize resolution (Cohn, 1969). Solution for washing was chosen according to the stain which was to be employed. For example, washing the preparation with an alcohol was preferred when an alcoholic staining solution was to be used (Peacock, 1958; Jensen, 1962). Washing solutions are included with details of staining.

Sometimes during preparation and particularly during the dehydration steps, the sporangiophores became curled and determining the distribution of organelles was difficult. For this reason and also for minimizing the effect of mechanical manipulation on the distribution of cytoplasm, sporangiophores were linearly fixed with egg albumin (see appendix) with their long axis straight. A clean and dry slide was rubbed with a drop of the egg albumin. Sporangiophores were gently lowered on to the slide and a drop of water was also added. Sporangiophores were carefully straightened on to the slide with the help of a tooth pick or a fine needle. The preparations were dried in air (15-30 minutes) at room temperature  $23 \pm 2^{\circ}\text{C}$ . The preparations then can be subjected to the following steps of cytological procedures.

### 2.3 Staining of nuclei

The following dyes were used for staining fixed sporangiophores:

1. Feulgen stain
2. Giemsa stain
3. Acridine orange stain

The preparations of these dyes and of the other related cytological solutions are described in the appendix.

Methods used for staining sporangiophores are described below.

# 1. Feulgen stain

The method discovered by Feulgen-Rossenbeck and described by Robinow (1957), Davison (1968a,b), Hawker et al., (1968) was used for staining DNA. Sporangioophores were fixed in various fixatives (see above) for 1 hour after being treated to remove the cuticle. They were washed in distilled water for 10 minutes and then they were hydrolysed in 1N HCl at 60°C for 8 minutes; the jars containing the acid solution were held at 60°C in a water bath. The preparations were immediately washed for 8 minutes in distilled water after hydrolysis, to remove any acid excess. This was followed by placing the preparations for 90 minutes in a sealed jar containing stain solution which was kept in the dark. Preparations were then washed in sulphur dioxide water (see appendix) for 15 minutes, until excess stain was removed, except for that bound in the nuclei. At first attempt this period was checked, under the microscope to ascertain the time which was required for obtaining optimal staining of nuclei. The sporangioophore preparations were dehydrated in a series of alcohol solutions of strengths 20%, 40%, 60%, 80%, 95% and 100% absolute ethyl alcohol for 1 minute each. Then they were cleared in xylol for 1 minute and mounted in D.P.X. Canada

balsam, glycerine and euparal were also used as alternative mountants, but with experience, D.P.X. was preferred.

Acetic acid-alcohol in a ratio of (1:3 v/v) gave the best preparation and thus was preferred as a suitable fixative.

## 2. Giemsa stain

The Robinow's (1957) technique was used for staining sporangiophore in the present work.

Sporangiophores were fixed in several fixatives for 1 hour each. They were washed in distilled water for 10 minutes, then hydrolysed in 1N HCl at 60°C for 8 minutes. The preparations were washed immediately in distilled water for 10 minutes to remove the excess acid. This step was followed by placing the preparations in jars containing diluted stain (1 ml of concentrated stain solution was diluted with 10 ml of 0.1M phosphate buffer at pH 7.0) for 1 hour. Then they were washed in distilled water for 6 minutes to remove the excess stain. Dehydration and mounting were as for Feulgen.

Temporary mounts were also made in a drop of dilution buffer with the edges of the cover slip sealed with vaseline or nail varnish. Acetic acid-alcohol fixative was usually used in preparations for this staining method.

### 3. Acridine orange

This stain was used for staining both DNA and RNA (Jensen, 1962) and the same method described was also used in this work. The staining solution was prepared (see appendix) using 0.1M phosphate buffer at pH 7.0.

Sporangiophores were fixed as usual in most of the fixatives for 1 hour and then washed in the buffer solution for 10 minutes. They were stained in the stain solution for 30 minutes, then washed in the buffer for 5 minutes. Following mounting as described above, specimens were examined under a fluorescence microscope at wavelengths 400-490 nm. Nuclei stained a green to yellow colour while RNA stained as a red background.

### 2.4 Staining of RNA, DNA and protein

Several stains were employed such as toluidine blue, pyronin Y, acridine orange (for RNA), methyl green, Feulgen (for DNA) and orange G (for protein) but they gave similar observations as Korson's stain (Korson, 1951). Korson's stain is a triple stain and can be used for determining RNA, DNA and protein in the same preparation; therefore it was preferred and used extensively in the present work.

#### 1. Korson's or Triple stain

Procedure based on Korson's (1951) method was used

as a specific and, at the same time, differential stain. It was specific since a component of the stain is specific for a definite macromolecule, and differential in that it can be used to show the three macromolecules in one sample. Korson's stain consists of three solutions:

- A - 4% orange G in distilled water
- B - 0.15% methyl green in distilled water
- C - 1 part of 0.1% toluidine blue in distilled water  
plus 1 part of 0.15% methyl green in distilled water.

Sporangiophores were fixed in acetic acid-alcohol fixative for 1 hour, then the preparations were washed in distilled water for 5 minutes. They were placed in solution A for 2 minutes, and then washed in distilled water to which was added 1 drop of solution A, followed by placing the slides in B solution for 15 minutes.

Solution B was then drained off and the slides were left in solution C for 5 minutes. Finally the preparations were rinsed in tertiary butyl alcohol or, more usually, ethyl alcohol (100%) and left for complete destaining overnight (see Hepden & Hawker, 1961; Jensen, 1962; Hawker et al., 1968; Davison, 1968a,b). Following the destaining step, the preparations were cleared in xylol and then mounted in D.P.X. for permanent preparations. Examination was undertaken by transmission and phase

contrast microscopy. DNA was stained green in colour, RNA blue and protein was stained faint red in colour.

## 2. Distinction between DNA and RNA

In some preparations of Korson's stain, recognition or distinction between RNA, DNA and protein was needed (Jensen, 1962). Superimposition of one stain on to another has been shown to cause some difficulties in recognizing the exact location of the macromolecules. To distinguish between RNA and DNA, use was made of both DNAase and RNAase. 16 sporangiophores were divided into 4 groups and each group consisted of 4 sporangiophores. The first group was treated with 0.1% RNAase solution of pH 6-8 at 37°C using a water bath. The pH value was adjusted with a minimum volume of 0.1M NaOH in the absence of any buffer. The sporangiophores were incubated in the solution for 90-120 minutes, then they were washed in water and stained for RNA, DNA and protein by Korson's stain. No dark blue colour indicating RNA was observed.

The second group was incubated in a solution consisting of 0.2 mg.ml<sup>-1</sup> DNAase in 0.003M MgSO<sub>4</sub> solution of pH 6.5 at 25°C in a water bath. The incubation period was 1-2 hours. NaOH was used to adjust the value of the pH. After washing DNA was stained by Korson's stain. No green colour was observed in these sporangiophores.

The third group was placed in water at pH 6.5, and used as a control. The fourth group was treated with both RNAase and DNAase respectively. They were washed and stained for DNA, RNA and protein by Korson's stain. These sporangiophores showed only protein. This confirms that the staining of sporangiophores with Korson's stain is a true specific biochemical interaction with RNA, DNA and protein present in particular regions of the sporangiophore.

#### 2.5 Staining of mitochondria

Mitochondria of sporangiophores were stained with blue-tetrazolium stain by the method described by Jensen (1962), Davison (1968a,b), and Hawker et al., (1968). Blue-tetrazolium is a vital stain which is specific in interaction with succinic dehydrogenase which is found in the mitochondria. Unfixed sporangiophores were employed, since it has been found that the fixatives may inactivate the enzyme and thus unsatisfactory results would be obtained (see Jensen, 1962). Sporangiophores were fixed by freezing them in solid CO<sub>2</sub>, then they were placed in jars containing blue-tetrazolium solution (10 mg blue-tetrazolium in 10 ml of 0.01M phosphate buffer pH 7.2) for 20 minutes at 35°C. The sporangiophores were washed in distilled water for 8 minutes and mounted in distilled water.



### 3. Methods for determining the contents of walls produced during growth and regeneration of sporangiophores

Use was made of specific fluorescent stains, which bound to wall precursors, Peberdy (1978). The fluorescence stains used were Uvitex BOPT and Uvitex BM (supplied as 50% solution) and Photine (supplied as solid state). These fluorescent brightening agents were supplied to Dr. Pearson from CIBA GIEGY Company.

#### 3.1 Preparation of stain solutions

As a preliminary test, it was shown that maximal absorption was at 350 nm. 0.1% solutions in 0.1M phosphate buffer at different pH values were prepared to establish suitable pH for optimal absorption; pH 6.5 was found to give an optimal absorption of fluorescent stain.

The effect of light on the stability of stains was tested, by exposing a solution at  $100 \mu\text{g}.\text{ml}^{-1}$  of each stain to daylight for different periods (see Figure 2). Times were plotted against optical densities at a fixed wavelength of 350 nm. Uvitex BOPT showed more stability after 8 minutes than both Uvitex 4 BM and Photine. Thus precautions were taken to keep them in the dark by covering the bottles of the stock solutions. The flasks which contained a diluted solution were also covered with foil.

Uvitex BOPT only was used in the present study.

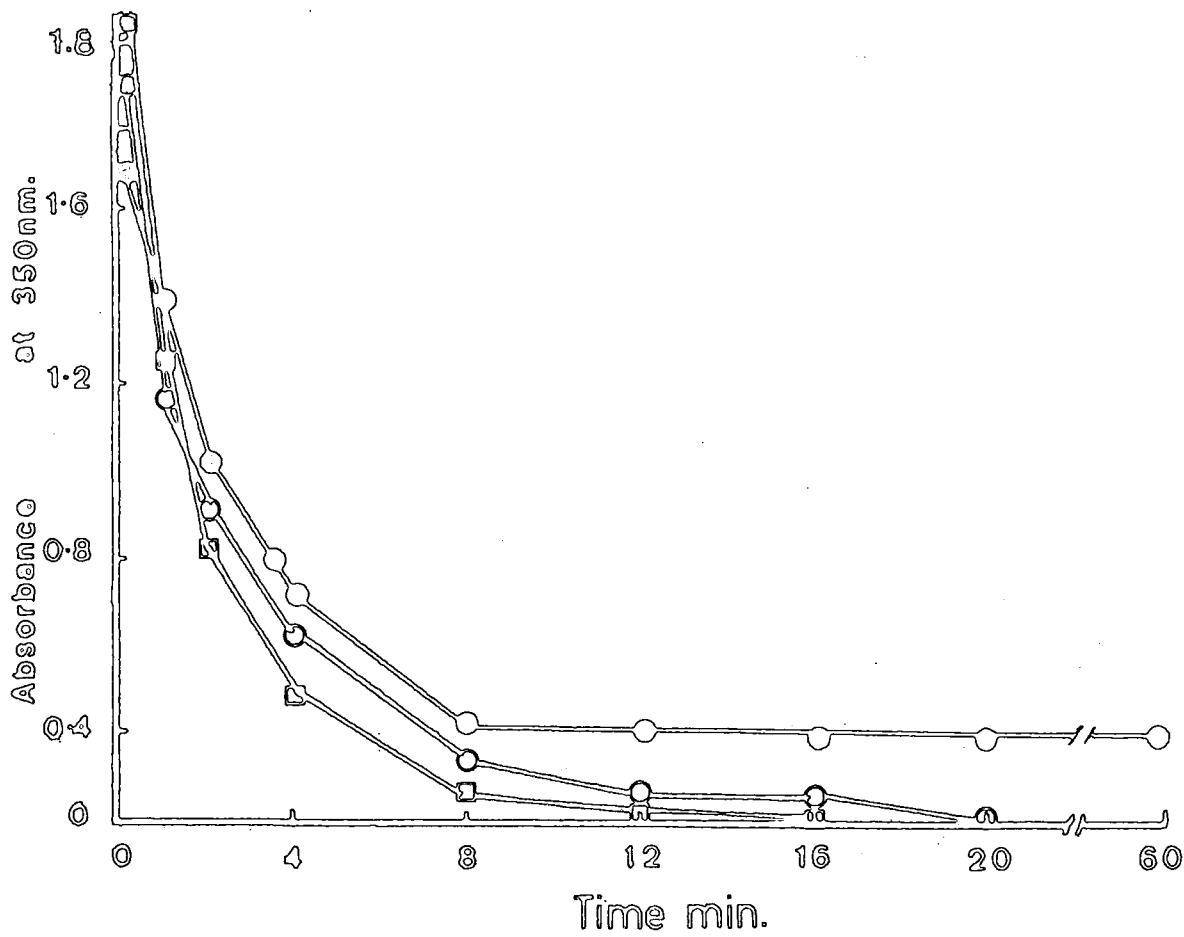
Figure (2)

Stability of wall stains, during exposure to day  
light for different periods

○ Uvitex BOPT

○ Uvitex 4 BM

□ Photine



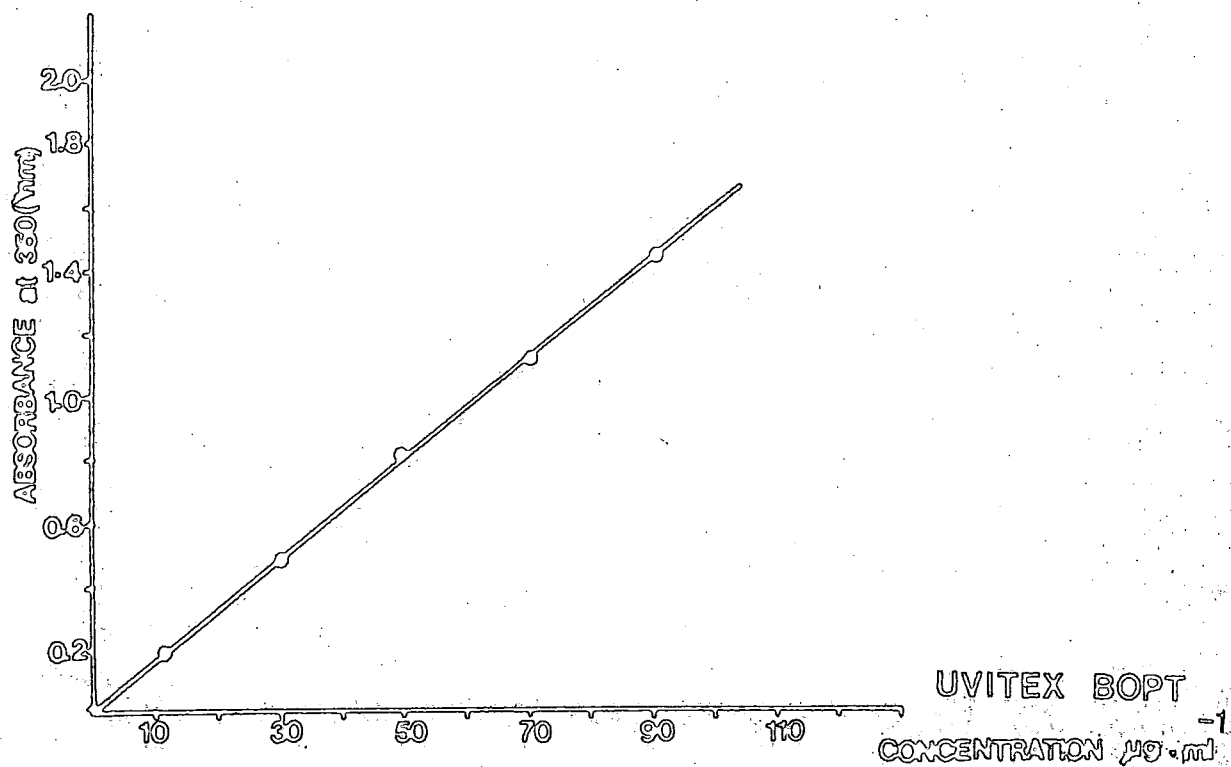
Solutions covering the range between 1-100  $\mu\text{g}.\text{ml}^{-1}$  were prepared in 0.1M phosphate buffer at pH 6.5. Optical density (O.D.) of each concentration was measured at 350 nm fixed wavelength, using the Unicam 1800 spectrophotometer. Calibration curves were drawn between the concentrations and optical densities (see Figure 3) and a linear relationship was obtained. Because of their high sensitivity to pH, light and temperature, an optimal condition for achieving a maximal stain adsorption by the wall of sporangiophore was determined.

### 3.2 Effect of the pH of the brightener solution on the adsorption

0.1% solutions of the Uvitex BOPT in 0.1M phosphate buffer at different pH values were prepared, covering the range between 5.8 - 9.0. Dried walls of sporangiophores were prepared from 1 gm fresh weight of sporangiophores of stage I, grinding them with a pestle-mortar after freezing in liquid nitrogen. The material was washed repeatedly with water and centrifuged at 500 x g until no more turbidity was seen in the supernatant. The wall was then collected from the tube and dried in a 55°C oven for 1-3 minutes. 0.5 mg of dry weight was placed in tubes each containing 1 ml of stain at different pH values. The quantity of the stain adsorbed by wall was calculated as follows:

Figure (3)

A calibration curve of Uvitex BOPT in 0.1 M phosphate buffer and at pH 6.5, for adsorption determination of sporangiophore wall.



The absorbance of the stain without added sporangiophore wall was used as standard. Solutions containing the wall material were centrifuged at 500x g and the supernatant dye decanted. The sedimented wall was washed three times with distilled water, until no more stain was released and the washings added to the dye decanted from the wall material. The absorbance of this solution was taken at 350 nm and corrected to allow for dilution. The amount of stain was calculated from the calibration curve which is shown in Figure (3).

The amount of stain adsorbed by the wall =

The amount of the stain given by the control-

The amount of stain given by those mixed with the wall.

The amount of stain adsorbed by the wall treated with the solution at different pH values were shown in Figure (4(1)).

### 3.3 Effect of incubation period and temperature on the optimal adsorption

The optimal adsorption of stain by the sporangiophore wall was determined by using 0.1% solution of Uvitex BOPT in 0.1M phosphate buffer at pH 6.5. The absorbance of stain was taken at different intervals, Figures (4(3)) of incubation. The optimal amount of the adsorbed stain at different periods was determined from the calibration curve. Optimal adsorption of the stain by the wall was obtained at 4 minutes after immersion. Thus 4 minutes was

used during the protoplast studied, as an appropriate time to achieve an optimal adsorption of 0.1% solution of stain in 0.1 M phosphate buffer at pH 6.5.

Different temperatures of solutions (10-45°C) containing 0.5 mg dry weight of sporangiophore ml<sup>-1</sup> stain were used, in order to obtain a suitable adsorption of stain by the prepared walls of sporangiophore. Water bath at fixed temperature was used. The optimal adsorption has been shown, Figure (4(2)), to be achieved at a range between 30-35°C.

#### 3.4 Method for wall staining

In addition to the quantitative measurement of the amount of wall regenerated by the sporangiophore, the location of the regenerated wall was also determined by the same stain (Uvitex BOPT). Fixed sporangiophores were placed in 1 ml of stain solution of 0.1% in 0.1 M phosphate buffer at pH 6.5 and incubated at 30°C for 4 minutes. Sporangiophores were washed in the same phosphate buffer, and the preparations were covered with a coverslip. The edges of the coverslip were sealed with a petroleum jelly and coated with a nail varnish. The preparations were examined with an ultraviolet microscope (at 260 nm wavelength).

All of the preparations were examined under transmission and phase contrast.



Microscopically, except those sporangiophores stained with acridine orange, they were examined with an incident light fluorescent microscope, using an appropriate wavelength excitation at a range of 400-490 nm. An automatic camera (Leitz) fitted on the microscope was used for recording results. The preparations were photographed in colour and in black and white films with a very high speed sensitivity (Kodak, 400 and 600 Tri-x pan films).

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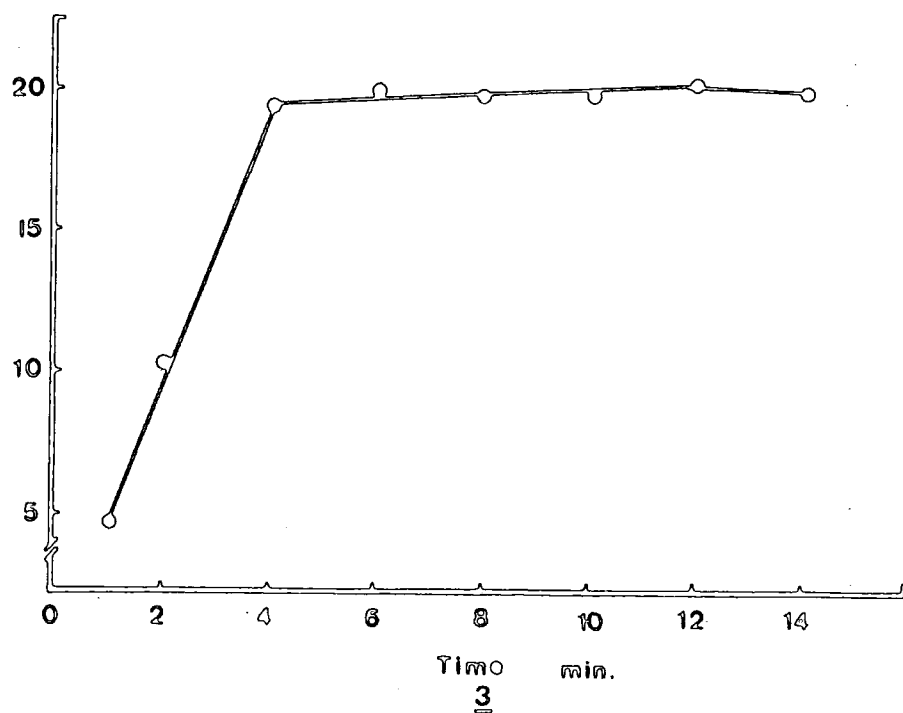
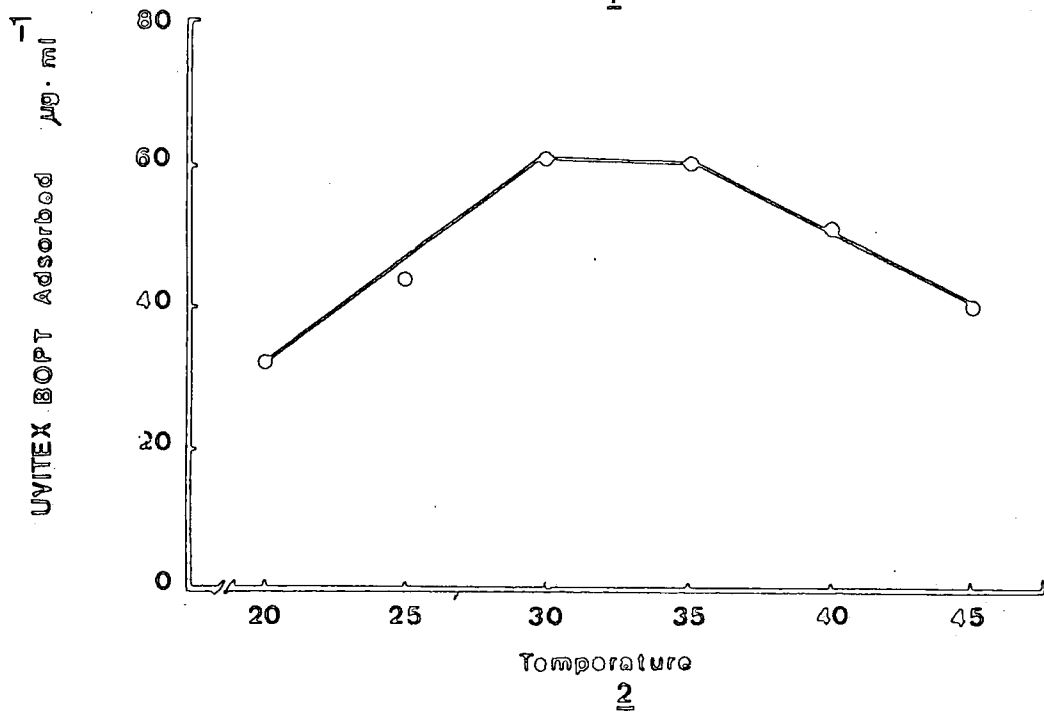
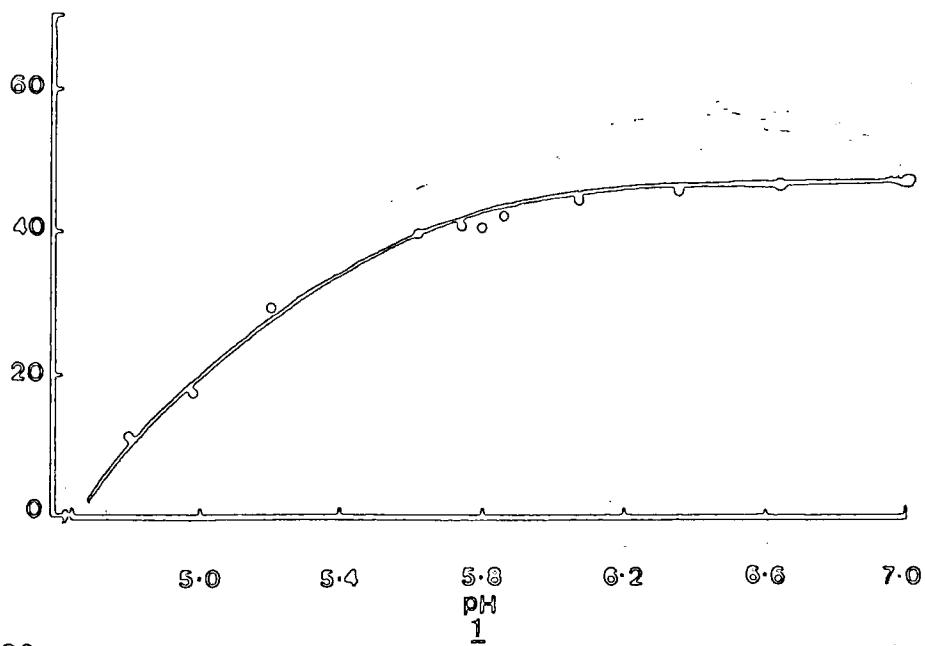
Figure (4)

Optimal adsorption of Uvitex BOPT by the walls of  
Sporangiophores (0.5 mg dry weight per 1 ml stain)  
at different conditions

1 - After immersion in the stain at different pH values  
of phosphate buffer (0.1 M)

2 - After immersion in the stain at pH 6.5 of 0.1 M  
phosphate buffer but at different temperatures

3 - After immersion in stain at pH 6.5 of 0.1 M  
phosphate buffer but at different periods of  
incubation



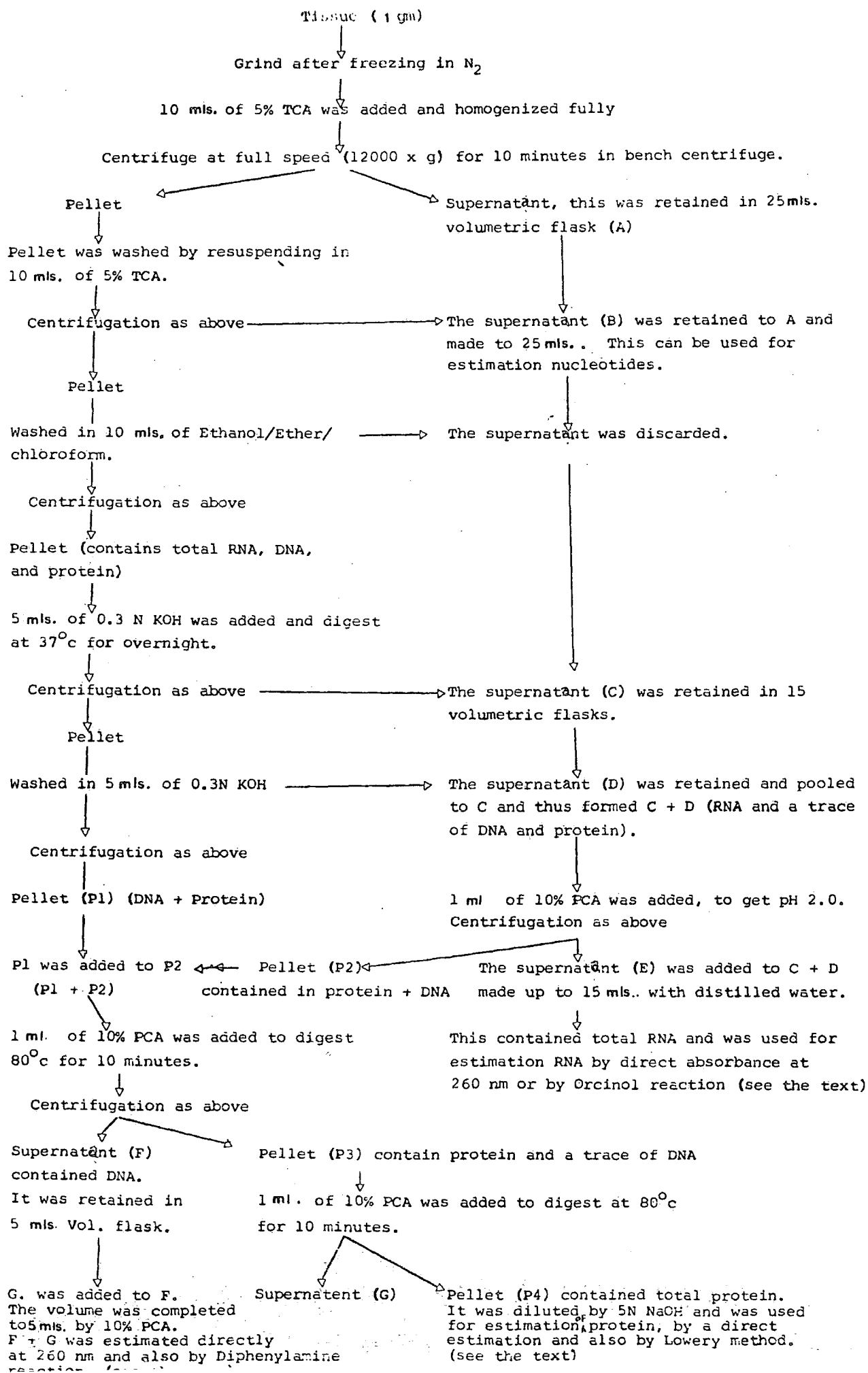
#### 4. Extraction and estimation of nucleic acids and protein

Methods described by Pearson (1969) and Storck & Morril (1977) were used. The same methods were employed for both sporangiophores and protoplasts. In the case of sporangiophores these were harvested when 15-20 mm in length (c. 1 gm fresh weight) and were washed with 0.01 M phosphate buffer pH 7.0. The extraction methods are illustrated in Figure (5).

Sporangiophores were frozen with liquid air and homogenized with a pestle and mortar, 10 ml of 5% TCA was added to the ground tissue and a full homogenization was carried out. The mixture was centrifuged at full speed in a MSE bench centrifuge for 8 minutes at room temperature. This was repeated twice and the supernatants (A + B) containing nucleotides were pooled and retained in a 25 ml volumetric flask. The pellet was washed by resuspending in 10 ml 5% TCA and centrifugation at 12000 x g for 8 minutes was carried out. The supernatant (B) was retained and pooled with that from A. A + B solution can be used for the quantitative estimation of nucleotides. 10 ml of ethanol/ether/chloroform (3:2:1 v:v:v) was added to wash the pellet (remaining pellet of B supernatant which should contain lipids RNA, DNA and protein), the mixture was shaken vigorously and then centrifuged as

Figure (5)

Diagram showing ways or procedures for extraction and estimation of macromolecules (DNA, RNA and protein)



above. The supernatant was discarded and 5 ml of 0.3N KOH was added and the mixture digested at 37 °C for 18 hours. The digested mixture was centrifuged as above and the supernatant (C) was retained in a 15 ml volumetric flask. Another 5 ml 0.3N KOH was added to the pellet and centrifugation was carried out; the supernatant (D) was retained and pooled with (C) fraction. The pellet here (P) can be used for DNA and protein extraction. C and D solutions (10 ml) were pooled. C + D should contain total RNA and a trace of DNA and protein; C + D solution was treated to precipitate the remaining protein and DNA. This was carried out by reducing the value of pH of the solution to pH 2.0 by adding, 0.51 ml 10% PCA. The suspension was centrifuged as above and thus the supernatant (E) was pooled to C + D and was used for RNA estimation by a direct absorbance at 260 nm wavelength, or by orcinol reaction. After centrifugation, pellet P2 was added to the pellet P1 (P1 + P2). P1 + P2 was digested with 1 ml 10% PCA at 80 °C for 10 minutes. This should precipitate protein from DNA, and centrifugation repeated twice and the F + G supernatant (2 ml) retained and pooled in a 5 ml volumetric flask (made up to 5 ml with 10% PCA). This was put in the spectrophotometer to estimate DNA or was used for the diphenylamine estimation of DNA. The remaining pellet, (P3), obtained after the



final centrifugation, was manipulated to extract and estimate total proteins. This was carried out using Lowry reaction (see below). During the extraction of nucleic acids and protein from the protoplasts, similar methods were used.

### 1. RNA

The orcinol method of Markham (1955) as described by Pearson (1969) was used for the present work for estimation of impure RNA. A solution of 40 ml conc. HCl + 10 ml 1% orcinol solution + 1 ml 10%  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ , was made up fresh each time. 2 ml of the stock solution was added to 0.2 ml of the RNA solution and heated in a boiling water bath for 9-10 minutes. 5 ml of n-butanol were added to each tube which diluted and stabilized the reaction mixture to a suitable extinction for measurement in a Unicam 1800 spectrophotometer. A reagent blank was used. The concentration of the RNA was calculated from a calibration curve which was constructed using adenosine 5' - monophosphate as a standard, Figure (6(1)). RNA in solution was also estimated using the absorption at 260 nm wavelength.

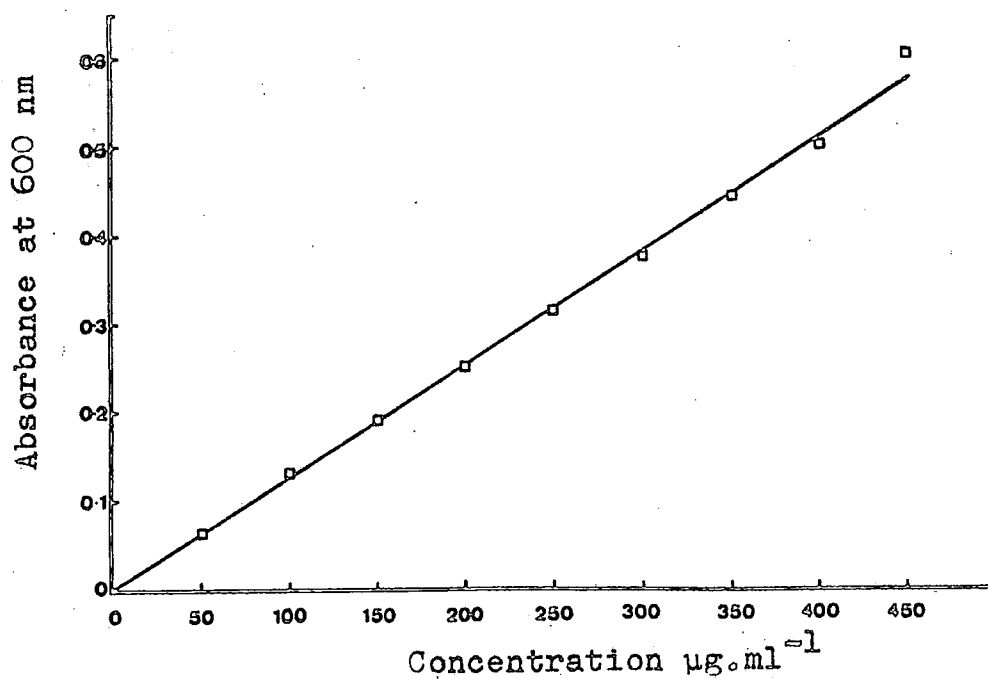
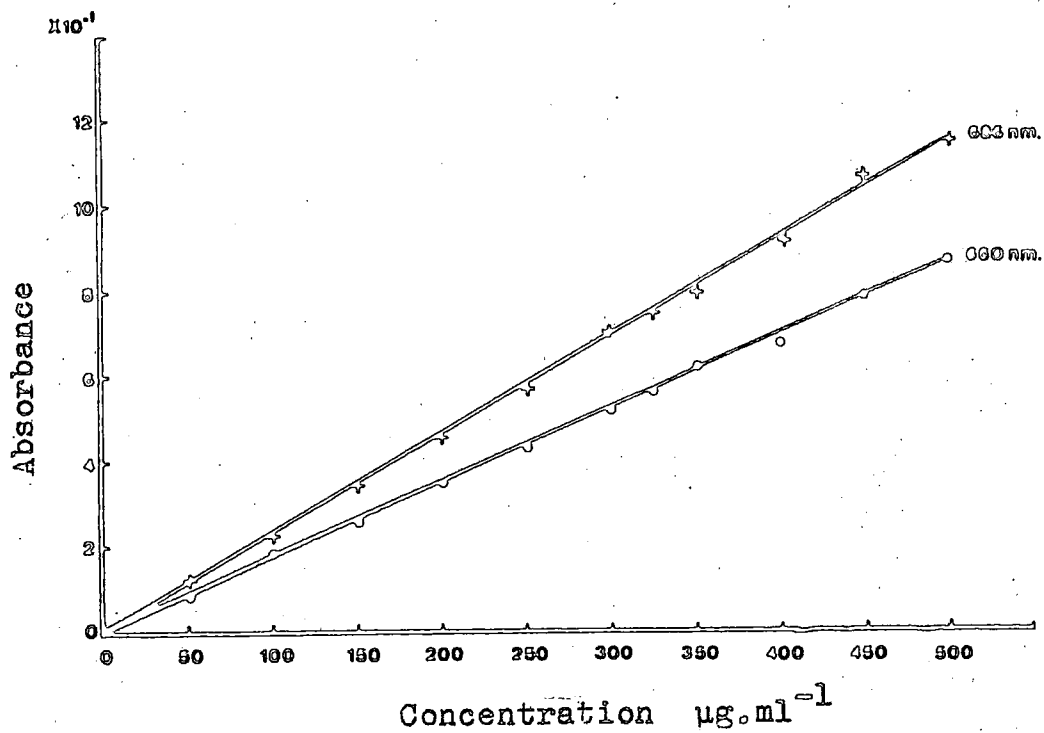
### 2. DNA

The diphenylamine test of Burton (1956) was used here. A stock solution of 1 gm of recrystallized

Figure (6)

1 - Calibration curve of AMP - 5 for orcinol  
determination for RNA

2 - Calibration curve of Calf Thymus DNA for  
diphenylamine determination.



diphenylamine in 100 ml glacial acetic-acid plus 2.75 ml conc.  $\text{H}_2\text{SO}_4$  was prepared. 0.2 ml of DNA solution was added to 0.05 ml perchloric acid (9.19 M) and 0.08 ml  $\text{H}_2\text{O}$  and heated at  $70^\circ\text{C}$  for 45 minutes. After cooling 2.1 ml diphenylamine reagent plus 0.1 ml of  $16 \text{ mg.ml}^{-1}$  acetaldehyde solution was added. Following incubation at  $37^\circ\text{C}$  for 18 hours, the  $E_{600 \text{ nm}}$  was measured for the sample. A calibration curve was made up using calf thymus DNA (absorbance was measured at  $E_{600 \text{ nm}}$ , Figure (6(2))). The concentration of DNA, of the tissue was calculated from the calibration curve.

### 3. Protein

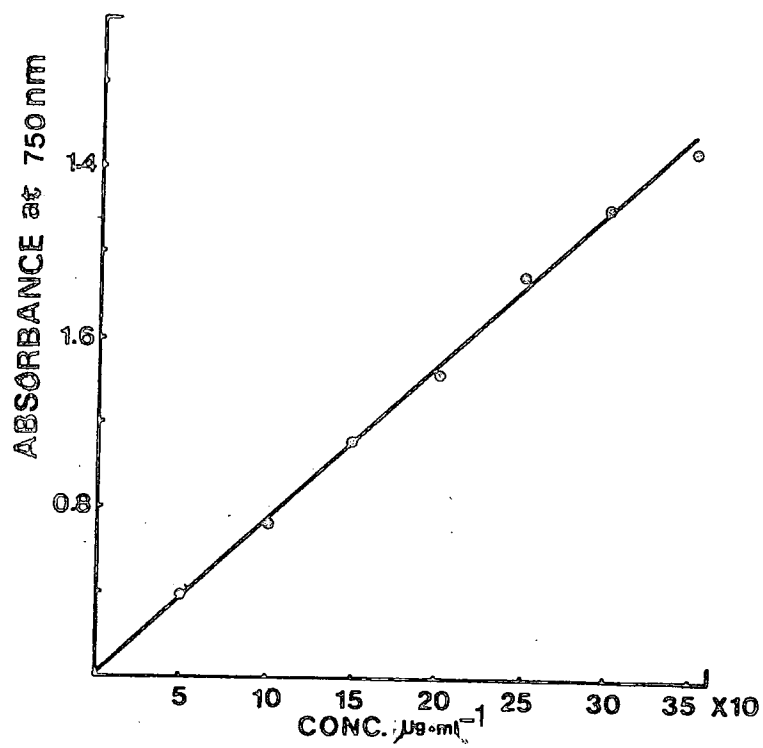
The method of Lowry et al., (1951) as described below was used to estimate protein. Two reagents were used:-

Reagent A: Mix together 0.5 ml of 1%  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  and 0.5 ml of 2% potassium tartarate. This was added slowly with stirring to 50 ml of 2%  $\text{Na}_2\text{CO}_3$  in 0.1 N NaOH.

Reagent B: Folin-ciocalteau reagent was prepared by diluting with 4 ml to 10 ml distilled water. Standard crystalline Bovin Serum Albumin (BSA) was prepared from  $5 \text{ } \mu\text{g.ml}^{-1}$  to  $350 \text{ } \mu\text{g.ml}^{-1}$ .

Figure (7)

Calibration curve of Bovin Serum Albumin for Lowry  
determination method for protein



Samples of protein (P3) were diluted with 5 N NaOH to approximately  $0.5-1 \text{ mg.ml}^{-1}$  protein. After mixing, this was left at room temperature for 10 minutes. 0.3 ml of reagent B was added and care was taken to ensure good mixing.

After 1 hour the optical density of the blue colour was measured at  $E_{750}$  nm wavelength against a reagent blank. A calibration curve of BSA was constructed, Figure (7). The concentration of protein of tissue was calculated.

## CHAPTER I

### Results

#### 6. GENERAL OBSERVATIONS

Obtaining synchronized growth in culture was a major objective to be achieved for studying growth and development of Phycomyces blakesleeanus sporangiophores. Following extensive investigation, it was noticed that the age of spores proved to be an important factor controlling synchronization. Use of 1-2 months old spores was found to reduce synchrony. Use of 1-15 day old spores and extension of the period of heat activation (normally for 6-8 minutes at 48°C) to over 30 minutes, was found to reduce the rate of germination and synchrony of subsequent sporangiophore development.

Moreover, age plays an important role during the induction of the synchronization of sporangiophores from the mycelium. 48 hours old mycelium was preferred, while much older mycelium gave poorer growth and less synchronization of sporangiophore. With much younger mycelium (12 hours old) there was a delay in growth and reduced final yield of sporangiophores.

Observations on such variable growth and synchronization contributed a useful approach to an understanding of the primary growth regulation, which



will be mentioned later.

Nearly synchronized sporangiophores were obtained on liquid and solid minimal medium (see general material and methods). After induction it was possible to study individually the various developmental stages of the asexual reproductive structure.

The first recognized morphological event of asexual reproduction was the formation of the foot cells; these were induced by placing the mycelium, when it had been filtered off from the liquid culture, onto the surface of a liquid or solid medium. The foot cells were also formed nearly synchronously on the surface of limited nitrogen medium. Foot cells were also produced in the non-induced culture, but not synchronously. Using methods described, synchronized foot cells formation was obtained, Plate (2).

The foot cell appeared, Plate (2(2,3,4)), as a branch, formed from the axis of the parent hypha. Its morphological structure resembled a pear-shaped lateral swelling. Preparations of substrate hyphae and the foot cells, were fixed by acetic acid-alcohol (1:3 v/v) and stained in situ with toluidine-blue stain. Greater density of staining could be seen at the apical region of the foot cells. This may indicate possibly induction of

a preferential accumulation of RNA. The diameter and length of the induced foot cells were uniform, but were variable in non-induced cultures, Table (1).

The diameter and length was measured from the Plates (2(4)) & 3(1)). These values were divided by 400 (magnifications) and then calculated in  $\mu\text{m}$  instead of mm. The size of the foot cells at the margin of the uninduced colony differed from those further back along the leading hyphae. It appears that the size of the foot cells depends on the degree of maturation of the substrate hyphae. The size of the foot cells in the induced cultures was nearly equal at the margin and centre of the substrate hyphae.

The diameter of the main axis of the substrate hyphae, generally was greater for the induced culture. Values of 12-30  $\mu\text{m}$  in diameter were obtained for all the hyphae.

Table (1)

The average diameter and length of the foot cell  
of nearly synchronized and non-synchronized cultures.  
Each value is a mean of three replicates.

| <u>Synchronized culture</u>                                | <u>μm</u> | <u>Non-synchronized culture</u> | <u>μm</u> |
|--|-----------|---------------------------------|-----------|
| The average diameter of<br>tip regions of foot cells       | 10        | At the margin of hyphae         | 7.5       |
|  |           | At the centre of hyphae         | 5         |
| The average diameter of the<br>basal regions of foot cells | 5         | At the margin of hyphae         | 5         |
|  |           | At the centre of hyphae         | 2.5       |
| Average length of the<br>foot cell                         | 78        | At the margin of hyphae         | 37.5      |
|  |           | At the centre of hyphae         | 7.5       |

The diameter of the substrate-hyphae of non-induced cultures, appeared to be different at the margin (6.2  $\mu$ m) when compared with fully grown hyphae at the centre (13  $\mu$ m) of the colony. The initiation of the sporangiophore from the foot cell as a tubular structure is shown in Plate (3(3)). The initial growth normally arose at the apex of the branched foot cell. These observations seemed to be different from the results that have been reported by Gruen (1959) who stated that the sporangiophore formation is not necessarily dependent on the formation of the lateral globular branches. In contrast to his report, observations here have shown that sporangiophores had their origins from branched foot cells; both the induced and non-induced mycelium, and this is in agreement with observations reported by Grehn (1932). Staining and different methods of culturing used in this study could be responsible for the different results observed differing from those of Gruen (1959).

During growth of non-synchronized cultures on the solid media, three regions of development of hyphae were seen, Plate (3(2)). Sporangiophores were shown to be initiated from the region 2, in Plate (3(2)). The hyphae at the centre of the culture were seen to have sporangiophores of stage IV, while no development of

sporangiophores was seen from the peripheral or marginal regions of tips of leading hyphae, Plate (3(2,1)).

Thus asynchrony of formation of foot cells during maturation of non-induced hyphae, could be attributed to asynchrony production of sporangiophores.

## 7. GROWTH AND DEVELOPMENT OF SPORANGIOPHORES

The developmental stages of sporangiophores are shown in Plate (4). Formation of the foot cells was followed by the initiation of an upward growing sporangiophore arising from the surface of the culture. This was considered in the present study as a second recognized phase of morphogenetic change in the development of the reproductive structure.

The upgrowth of an aerial hypha with a conical or tapered tip is generally designated as Stage I (Castle, 1942, 1959; Bergman et al., 1969; Trinci & Halford, 1975 and Burnett, 1976). This stage grew at a rate of  $0.3-0.5 \text{ mm} \cdot \text{hour}^{-1}$  and reached up to 80 mm in length by 12-15 hours after initiation, Figure (8). The final length of stage I has been shown to vary in different culture vessels. In petri dishes 60-80 mm in length was shown to be a maximum for stage I while heights of 180 mm were obtained from conical flask-grown cultures.

Stage II involved the modification of the apical growing region to give a globular swelling, with the migration of the cytoplasm to the growing region, resulting in the formation of a yellow sporangium. When swelling ceased there was a brief stage III of maturation when the colour of sporangium changed to brown. Stage II and

FIGURE (8)

Growth curves of sporangiophores

- Attached sporangiophores
- ▽ isolated sporangiophores cultured in water
- ▽ isolated sporangiophores cultured on solid medium
- isolated sporangiophores cultured in liquid minimal medium

Each value is a mean of 3 replicates

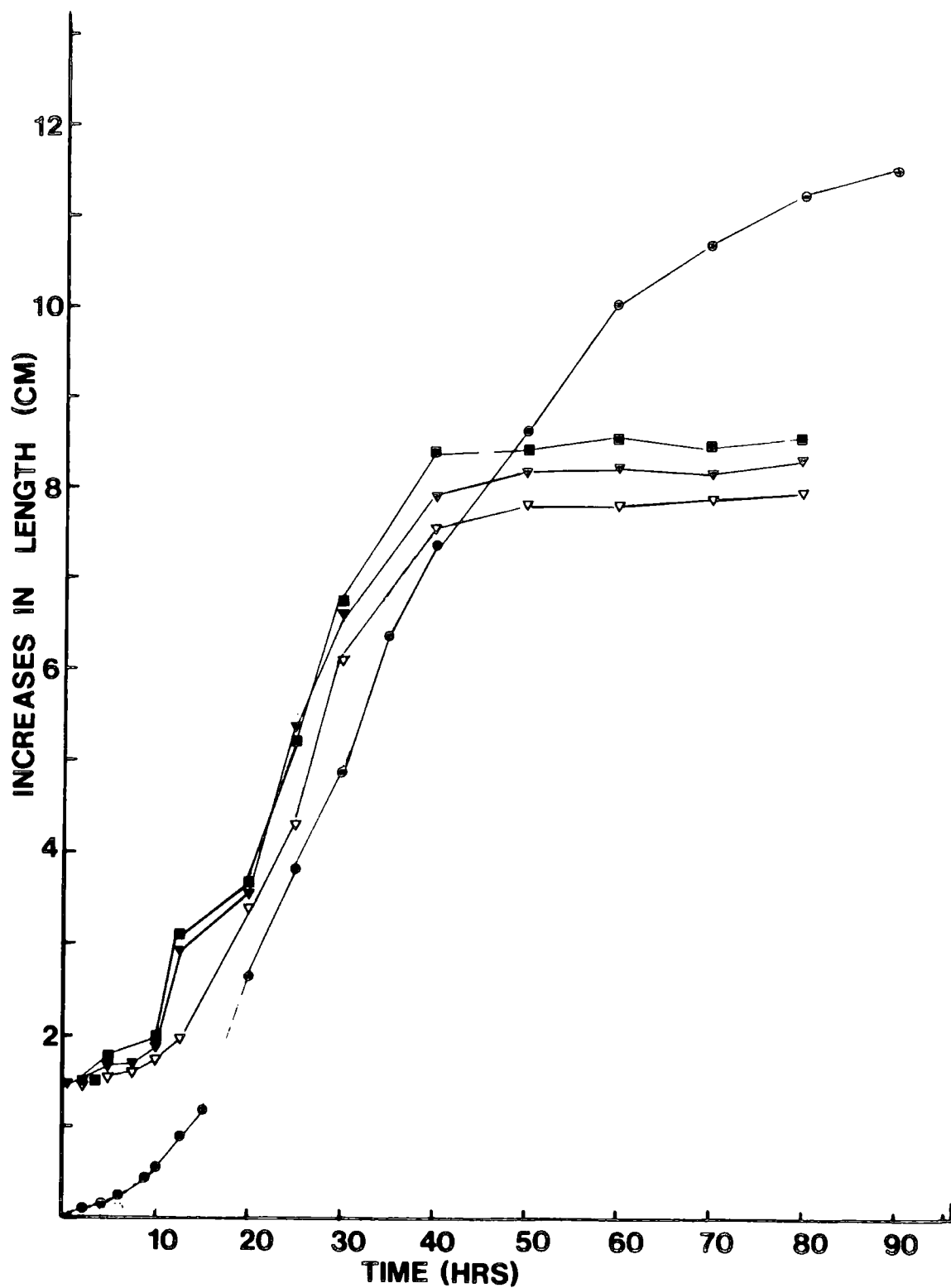
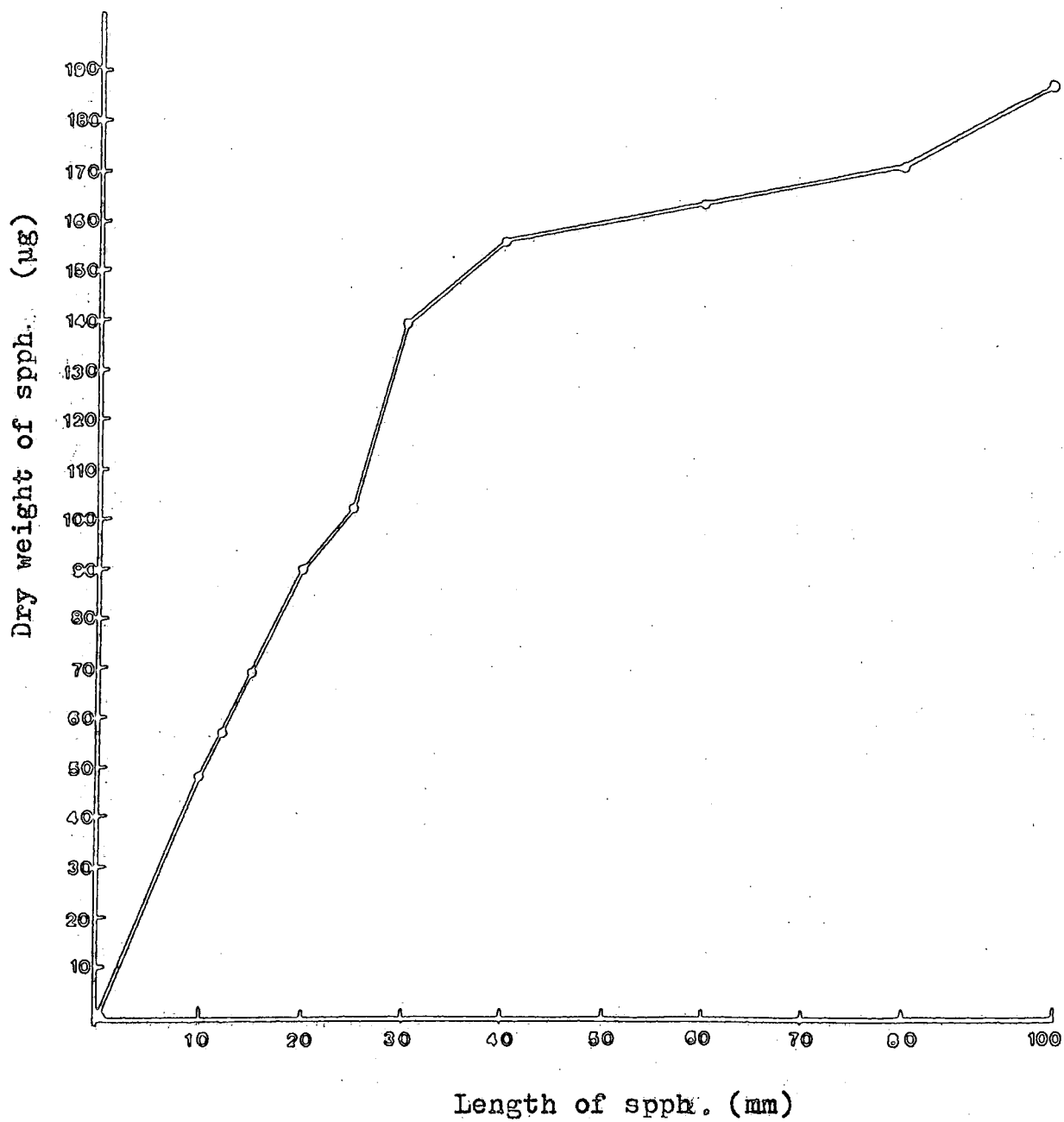




Figure (9)

Dry weights of sporangiophores of different lengths



III were achieved in a time between 14-16 hours after initiation of the sporangiophore (see Gruen, 1959; Castle, 1959; Burnett, 1976), with resumption of elongation in the inter-calary region below the sporangium, this passed to stage IV.

Sporangiophores at stage IV grew at a rate of 2.8-3.6 mm·hour<sup>-1</sup>, Figure (8). The final length of stage IV was also shown to be dependent on the vessel in which they grew. They reached 150 mm at 40 hours when they were grown in petri dishes, but reached 250 mm at 40 hours when they were grown in conical flasks. This may be attributed to the presence of more humidity in the flask, also the ratio between air/surface in the conical flask was greater than in the petri dish, which may have played a part in determining the growth of sporangiophore (see Bergman et al., 1969).

The increased length of stage I up to 40-60 mm was associated with a sharp increase in the dry weight, Figure (9). Dry weight increase was possibly due to increased migration of cellular components and cell wall formation. After sporangia formation, stage II, the sharp increase in dry weight ceased and at this stage it has shown that there is a cessation of migration of cytoplasm or nutrients from the hyphae (see Bergman

et al., 1969). In contrast when growth of sporangio-  
phores of stage I was up to 100 mm no sharp increase in  
dry weight was seen. This may be attributed to the fact  
that the translocation of the nutrients along that length  
from the substrate-hyphae, may have been impaired.

8. EXTRACTION AND ESTIMATION OF MACROMOLECULES DURING DEVELOPMENT

DNA, protein and RNA of stage I, II plus III and IV were extracted and estimated quantitatively as described in the materials and methods. The quantities of these macromolecules are shown in Figure (10(1)). The concentrations of DNA, protein and RNA were greater in stage I than in stage II plus III and IV respectively. The cessation of migration of the cytoplasm or nutrients from the substrate hyphae during the formation of stage II (sporulation) may be considered as a factor limiting the quantities of macromolecules formed during the subsequent developmental stages.

Macromolecules were also extracted from stage I sporangiophores of different lengths, Figure (11). Sporangiophores of 20-40 mm in length were shown to contain greatest quantities of DNA, protein and RNA. Increase in the protein during increase in the length may be attributed to the ageing or autolysis as previously described by Fenc1 (1978) for fungi in general.

Optimal increases of RNA and DNA which were found at 20-40 mm appeared to be correlated with the optimal

increase of the dry weight. During morphogenesis and sporulation of the sporangiophore, wall synthesis and distribution of nuclei were determined using Uvitex BOPT and acridine orange in combination as fluorescent stains. As shown in Plates (4(2), 5(2)), there is greater intensity of cell wall staining (Uvitex BOPT-binding) at the tip region of the stage I sporangiophore. During the transition from stage I to stage II by swelling of the tapered tip, the intensity of wall-component-specific stain was associated with the thickening wall. At this stage, nuclei appeared to be migrating into the swollen area, Plates (4(8,9), 6(1)). The migration appeared to take place, while the thickening was in progress, Plate (6(1,2,3)). More RNA staining intensity was also shown to be present in the sporangium after sporulation. During the maturation of the sporangium, less numbers of nuclei were found in the sporangiophore part below the sporangium. Otherwise a greater concentration of nuclei can be seen in the sporangium as shown in Plate (6(3)). Formation of the columella was found to be associated with cleavage of the protoplasm in the sporangium into megaspores, each of which appeared to contain several nuclei. Megaspore formation should be followed by cleavage into smaller spores (Thomas, personal communication).

Formation of the columella and megaspores were found to be associated with increasing intensity of the Uvitex BOPT in the wall of the columella and also at a point below the columella. Such observations suggest cell wall synthesis and growth extension in the intercalary region of the sporangiophore, Plate (6(4)). Additionally, nuclei were also shown to be abundant inside the columella. Thus, this may also be recognized as a special differentiation for intercalary growth extension of stage IV, and may reflect a significant physiological state of that region (Goodell, 1971).

The tip region of stage I appeared as a location of morphogenesis and differentiation during growth and development of the sporangiophore. Thus stage I of growth appeared to be an important step for determining the developmental processes and thus warranted further study. Most of the subsequent work reported here is in reference to changes taking place in stage I.

9. DISTRIBUTION OF CELLULAR COMPONENTS ALONG STAGE I SPORANGIOPHORES

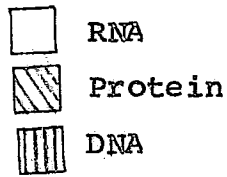
Further study of growth and development of stage I sporangiophores was carried out by extraction of DNA, protein and RNA from different regions (3-5 mm long). Each of the regions was of the same length and fresh weight, and were cut as tip, middle and base segments. The quantities of these macromolecules are shown in Figure (10(2)). Greater quantities of DNA, protein and RNA were found in the tip region of the sporangiophore.

In addition to the extraction study, nuclei, mitochondria DNA, protein and RNA were also determined cytologically using staining methods as described in materials and methods. The preparations are shown in Plates (5(2), 7(1,2,3), 9(1,2,3), 9). A greater concentration of staining for nuclei, mitochondria, DNA, protein and RNA can be seen at the tip region of the sporangiophore. The presence of RNA and DNA was confirmed by pre-treating the sporangiophore with DNAase and RNAase respectively (see materials and methods). Sporangiophores which were treated with DNAase were shown to stain intensely for only RNA only at the tip region with no evidence of DNA. Also those which were treated with RNAase showed intense

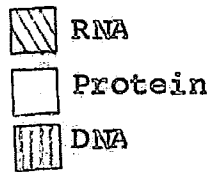


Figure (10)

1. Concentration of macromolecules extracted during growth and development of sporangiophores



2. Concentration of macromolecules extracted from different segments of stage I sporangiophores



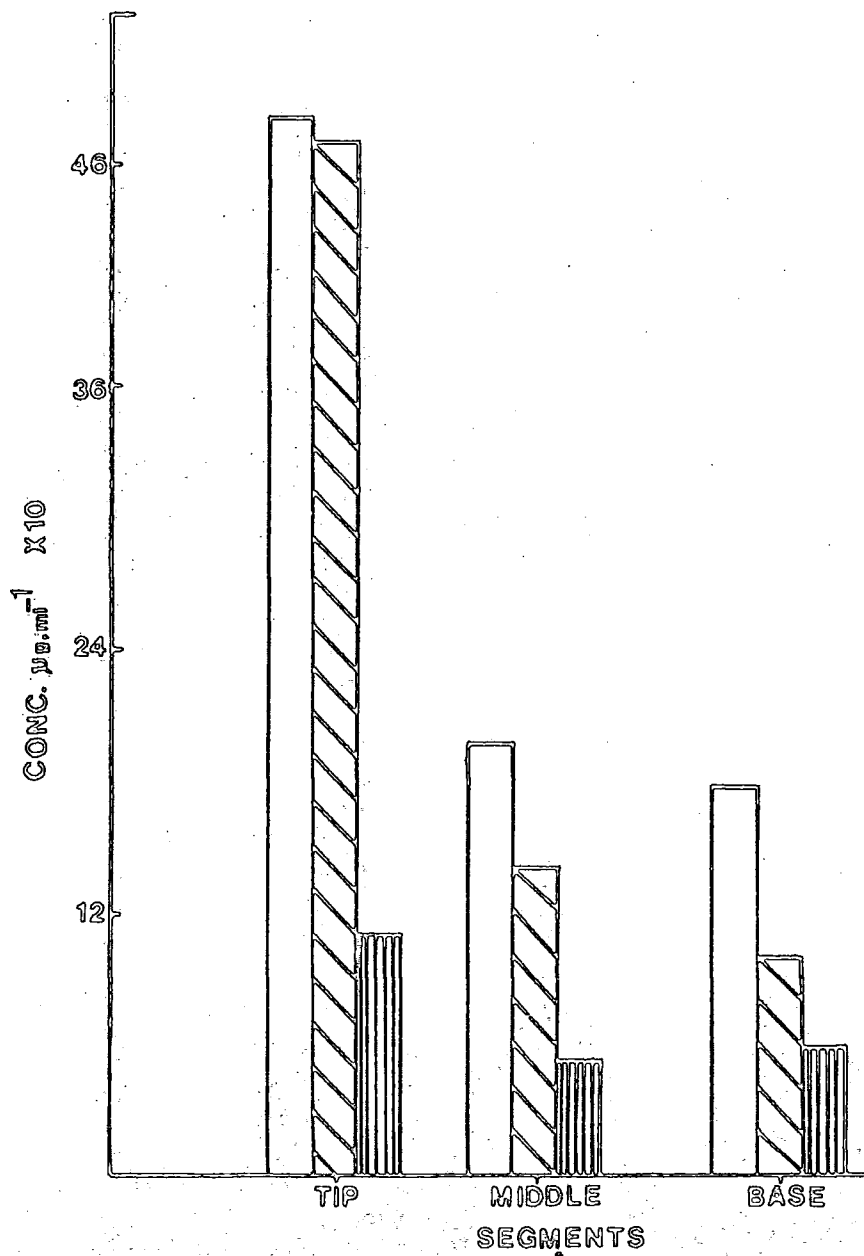
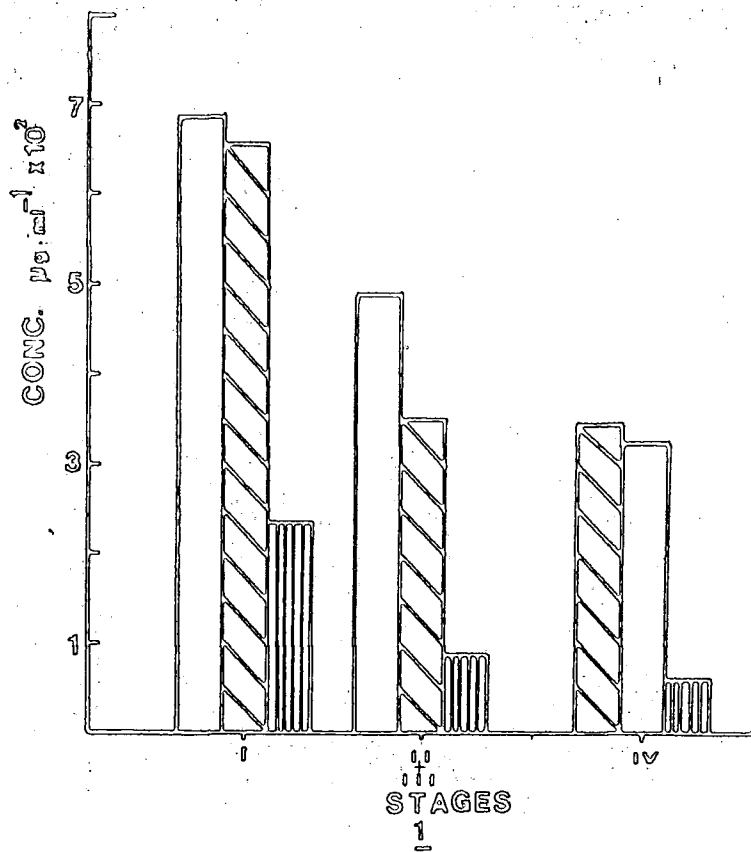
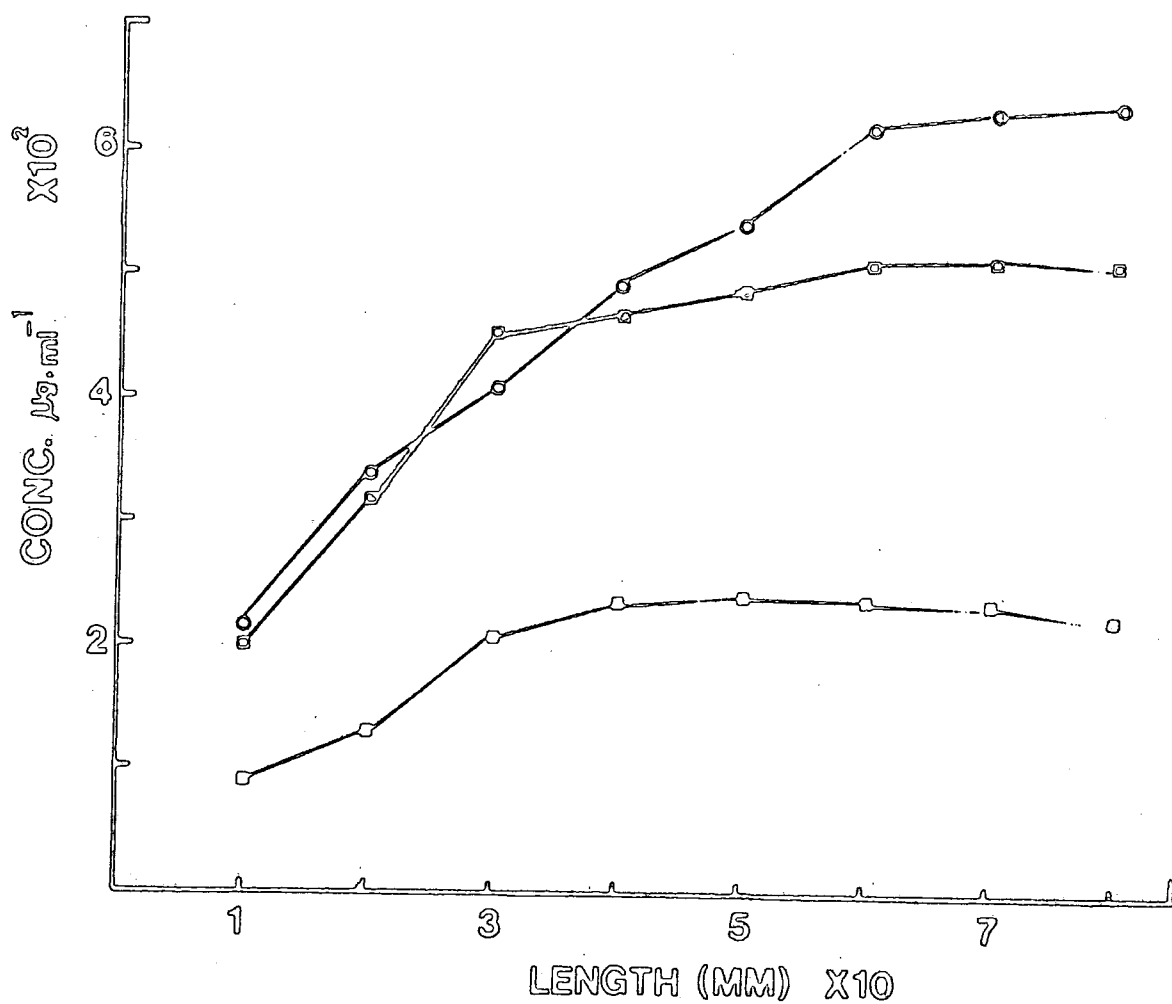


Figure (11)

Concentration of macromolecules extracted from stage I  
sporangiophores of different lengths

- DNA
- RNA
- Protein



staining for DNA without RNA, Plates (9(6), (7a)).

The extreme apex of the tip region was shown to be devoid of nuclei, Plate (10). These observations are in agreement with observations cited by (Bergman et al., 1969) and also resembled electron microscope studies by Peat & Banbury (1967), Hankinson (1972) who found a greater concentration of ribosomes, nuclei and mitochondria at a region below the extreme apex of the sporangiophore. The intensities of staining for RNA, DNA and protein were found to decrease with distance below the tip region of sporangiophore, Plates (5, 4(1), 5(2), 9(3a, b), 9(5b)). RNA and DNA were shown to be in greater concentration at the basal obconical region than in the middle region, but still at a level less than in apical region, Plates (9(2c), 10)). The intensities of staining for nuclei and mitochondria were also found to decrease with distance below the tip region, Plates (7, 8(1, 2, 3)).

From the above observation the stage I sporangiophore can be divided into regions according to the non-uniform distribution of cellular components, see Figure (12).

A - A region (1-2 mm in length) stained intensely with stain binding to the cell wall (see also Plate (18(1))). It also contained a greater quantity of protein, the presence of which may be attributed to the presence

of vesicles, since it was suggested that vesicles may carry cell wall enzymes and also include the building blocks for wall synthesis of fungi (Girbardt, 1969; Grove et al., 1970; Bartnicki-Garcia, 1973; Trinci, 1974; Weber & Hess, 1976). This region (A) resembled the tapered region named by Trinci & Halford (1975) and can be recognized as the growth extension zone (G-Z).

B - The sub-apical region which starts at a zone just below the base region of region A. This region was shown to stain intensely for RNA Plates (4(1), 9(19,3a)), mitochondria, Plate (7(2,3)), DNA, Plates (8(2),9(2a)), protein, Plate (9(1b)) and nuclei Plate (11). Its length was measured and was found to be between 2-4 mm with a diameter of 100-150  $\mu$ m. Distinction between A and B regions was not always possible due to over-staining but use of more than one stain allowed the recognition of the two zones. B region was also shown to contain a zone recognized as an exclusion zone (Thornton,1968b), which appeared as an empty central portion surrounded by cytoplasm containing many nuclei, Plates (10 & 11). The exclusion zone was not recognized in preparations stained for RNA and protein. Thus it is possible that the zone, although devoid of nuclei, contains high levels of ribosomes. The walls of the

sub-apical region stained intensely with cell wall fluorescent stain. A higher activity of wall synthesis has been found to be a characteristic of the corresponding region of the hyphae of some filamentous fungi (Chang & Trevethick, 1974). The tip region of the hyphae of filamentous fungi was considered as a younger region and was also characterized as a location of high metabolic activity (Mortimer & Johnson, 1959; Fencel, 1978).

In filamentous fungi, it has been suggested that both RNA and protein synthesis have higher activities in the sub-apical region (Zalokar, 1959a,b; Nishi et al., 1968). Zalokar, (1959b) concluded from his cytochemical technique that the apical growth of a Neurospora crassa hyphae was supported by a supply of precursors from a region of the hyphae at a considerable distance behind the tip. Likewise, it was envisaged that the wall of the sporangiophore tip was maintained in an extensible condition by a balance between wall synthesis and wall lysis, and that the driving force for wall extension is supplied by the turgor pressure of the protoplast, Trinci (1978b).

C - This region was characterized in that it contained the vacuole. Less intensity of stain and a

lower concentration of nuclei, mitochondria, DNA, protein and RNA were found to be associated with this vacuolation zone, Plates (9(2b, 3b, 5, 7b), 10)). This region was found to be initiated at a region below the B region and at approximately 4-5 mm from the extreme apex of the sporangiophore (at 15-20 mm in length). Diameter of this region was of the order of 160-180  $\mu$ m.

Increase in the size of the vacuole was found to be greater at parts more distant from the sub-apical region; at the same time there was a decrease in the concentration of the cytoplasmic organelles and components. The cytoplasm was only found in an area surrounding the vacuole since vacuolation left a very narrow peripheral zone (1-2  $\mu$ m thick) containing the cytoplasm and thus the increasing size of the vacuole could be associated with a decreasing volume of the cytoplasm. According to the concentration of the distributed cytoplasmic organelles and components, this region can be divided into zones designated as middle and basal regions, C & C' (Figure, 12).

The extreme obconical region of the sporangiophore (a region which connected the extreme basal end of sporangiophore with the substrate-hyphae) was found to contain greater intensity of the stains for nuclei, RNA and DNA. This may be attributed to its location, which

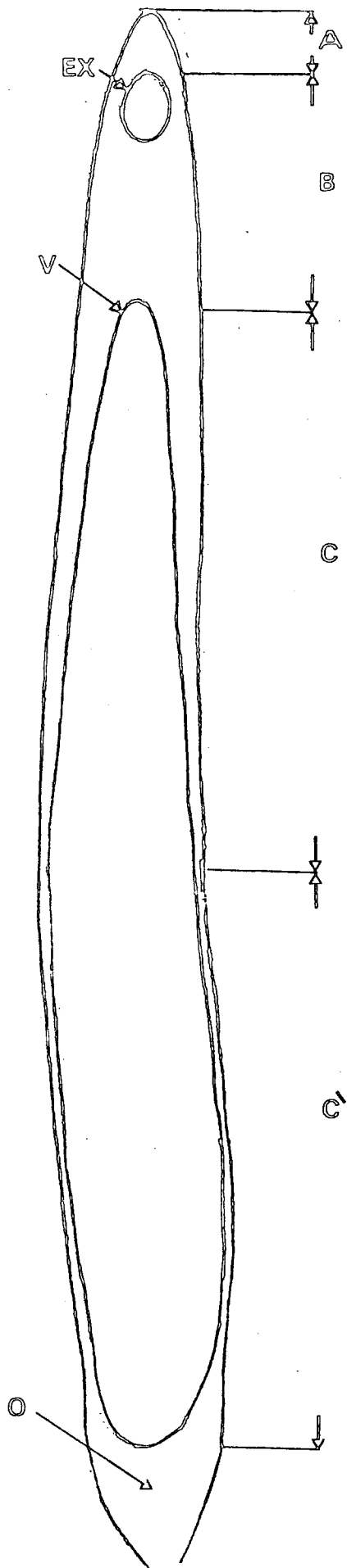


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Figure (12)

Sketched diagram showing the different regions along sporangiophore of stage I, according to the non-uniform distributions of cellular components

- A: Apex region
- B: Sub apex region
- C: Middle apex region
- C': Base region
- EX: Exclusion zone
- O: Obconical region
- V: Vacuole



is near to the substrate hypha. The substrate hypha have been found to support stage I sporangiophores with required substances (Bergman et al., 1969; Galpin & Jennings, 1976). Thus there is an inverted tapered region through which migration of organelles may take place from the substrate hypha.

## 10. ISOLATION STUDIES

### 1. Effect of isolation from substrate hyphae on the growth and development of full length sporangiophores

The fundamental interrelationship between sporangiophore and mycelium of Phycomyces blakesleeianus was examined by isolating the sporangiophore from the mycelium and culturing it on minimal medium (liquid and solid) and in water. The growth of these isolated sporangiophores was studied by measuring the increased length and dry weight at intervals. 16 sporangiophores at stage I and at initial length 10-20 mm formed a sample. The growth curves are shown in Figure (8). Each point represents a mean of 3 replicates. The overall pattern of the growth curve for attached and isolated sporangiophore was shown to be similar, but the isolated sporangiophore appeared to have a marginally longer lag-period before starting growth.

The isolated sporangiophores reached the same final stage of development as did the attached stage I sporangiophores however, the final length of the isolated sporangiophore at stage IV was less than that of the attached. Turgor pressure and substances supplied by the mycelium may have contributed to the increased length of attached sporangiophore. The attached sporangiophore showed

initially a strong erect cylindrical shape, while the isolated sporangiophores showed a loss of its cylindrical shape after the isolation and did not regain a normal form. It has been reported that the apical growth extension of the sporangiophore can take place through the effect of turgor pressure of the protoplast (Trinci, 1978b). Additionally continuous supplement of the nutrients from the mycelium during growth of attached sporangiophore at stage I (Bergman et al., 1969) can be used as an explanation for such differences.

There were no such differences in the final length of stage I of isolated sporangiophores (at the first 15 hours) which were cultured in water and in minimal medium. This may be attributed to the fact that the initial growth of isolated sporangiophore may not depend on the environment but it may be regulated by the reorganization or redistribution of the internal components, as suggested by (Gruen, 1967; Gruen & Ootaki, 1972). Reorganization of cellular components during growth of isolated fruit bodies of Coprinus cinereus was also suggested to maintain the wall synthesis (Gooday, 1974). It has also been suggested that during the starvation of fungal hyphae, the growth of hyphae can be maintained at the expense of the other compartments (see Trinci & Righelato, 1970).

Thus the autolysis or degradation of cellular components at parts below the tip region may contribute to the maintenance of the apical growth during the isolation or starvation as suggested recently by Fenc1 (1978).

Dry weight was found to be a constant value at the time of length increase, Table (2). Thus the growth of the isolated sporangiophore did not depend on the medium but appeared that it involved utilization of recycled cytoplasmic components as suggested above. The basal end of the sporangiophore which dipped into both water and minimal medium regenerated a mycelium, see Plate (12(2,4)). More regeneration was found at the basal ends of sporangio- phores which were dipped into minimal medium, Plate (12(2)) than in water, Plate (12(4)). No promotion of the growth of the sporangiophore was found after the mycelium regenerated. This might be due to the fact that the regenerated mycelia were submerged and not floating on the surface of the liquid medium. Submerged mycelium with a limited aeration may be unable to provide a sporangiophore with some requisite substance(s) which is produced to an adequate extent by better aerated hyphae at an air/liquid interface (see also Gruen 1967; Smith, 1978).

Table (2)

Dry weight of isolated sporangiophore (15 mm in  
initial length) during culturing

| Time (hrs) | 2    | 6    | 10   | 12   | 14   |
|------------|------|------|------|------|------|
| Dry weight | 68.1 | 67.5 | 66.2 | 67.2 | 67.6 |

The basal ends of sporangiophores which were cultured on the surface of the solid medium, regenerated directly to sporangiophores instead of mycelium, Plates (12(3), 13(1,2,3), 14(5)). Thus under aerobic conditions, sporangiophores were produced but under anaerobic conditions mycelium was formed. Such results are consistent with the observations, that aerobic conditions give an induction of synchrony of sporangiophores development.

The sporangiophores regenerated from the basal end of the isolated sporangiophore were shorter and narrower and formed smaller sporangia than those formed at the apical region, Plate (13(1,2)). More than one sporangiophore was found regenerated from the basal end of the full length sporangiophore, Plate (13(1)).

During regeneration, sporangiophores exhibited strong phototropic response to the head illumination, Plates (13(4), 14(4,5)). This appeared to be in combination with a weak geo-response since sporangiophores regenerated in the dark, Plate (13(3)), did not grow in a fully vertical fashion. Trinci & Banbury (1967) studied the growth of Aspergillus giganteus and reported a similar condition of a strong phototropic response to unilateral illumination, with no geo-sensitivity. The redistribution of the cellular



components during regenerating the growth of the isolated sporangiophore was investigated since it has been suggested that apical growth of fungi may be maintained with a continuous flow rate of cytoplasm and nutrients from the parts below the tip region (see Fenc1, 1978).

2. Re-organization and re-distribution of cellular components during regeneration and growth of sporangiophores

During growth of synchronized isolated sporangiophores which had tapered tips, DNA, protein and RNA were extracted and estimated as described previously. At different time intervals after culturing in minimal medium, sporangiophores were cut into three equal length segments (tip, middle and base). The above cytoplasmic components were extracted and estimated quantitatively, Figure (13). As shown during growth of sporangiophores the concentration of DNA, protein and RNA was decreased in the basal and middle region and correspondingly maintained in the tip region of the sporangiophore. The shift of DNA and RNA from the parts below to the tip region was shown to be associated with increasing quantity of protein and became more obvious during growth of stage II, where a lower concentration of components (RNA and DNA) was found in the middle of the sporangiophore than in the tip region. The increase in protein concentration at parts below the tip region could

Figure (13)

Concentration of macromolecules extracted from segments cut after growth of isolated sporangiophores in minimal medium and at different intervals

a.

Tip

- ☐ protein
- ☐ RNA
- ☐ DNA

b.

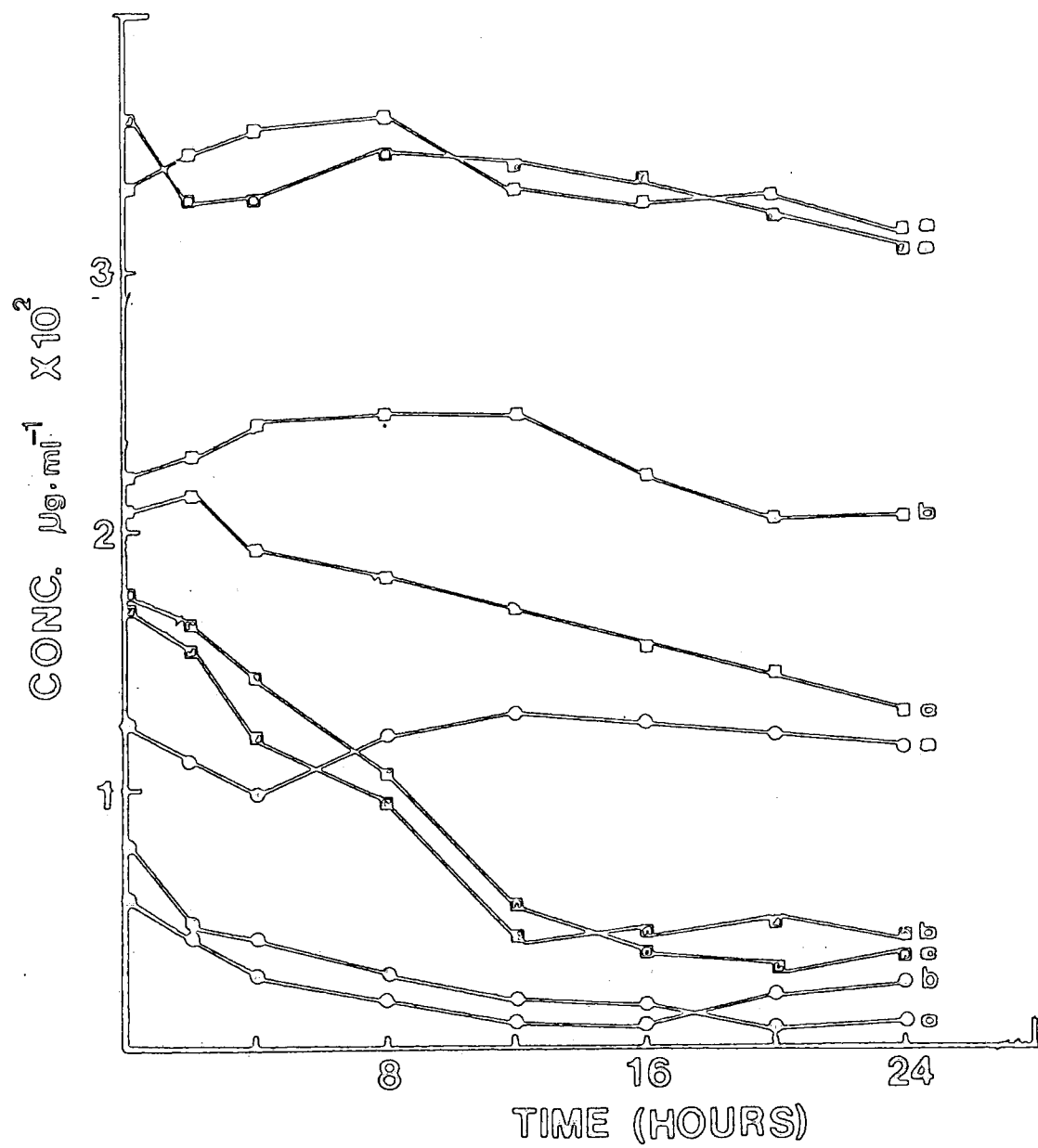
Middle

- ☐ protein
- ☐ DNA
- ☐ RNA

c.

Base

- ☐ protein
- ☐ RNA
- ☐ DNA



possibly be explained by an increase in some enzyme which may be involved in the degradation of the RNA and DNA. The initial decrease of macromolecules at the initial period of culturing could be attributed to the effect of mechanical shock. After growth resumed at the tip region (6-8 hours) RNA, DNA and protein were found to accumulate at the basal region of the sporangiophore. Such accumulation was followed by regeneration at the ends of the basal regions.

A decreased concentration of RNA and DNA in the middle regions of sporangiophores during the regeneration and growth of the isolated sporangiophores may be attributed to the internal adaptation between regions along the sporangiophore maintaining the normal level of concentration of macromolecules at a location of wall synthesis. This can occur by autolysis or degradation of macromolecules to simple compound which could be transported and utilized again in the tip or in the basal regions as precursors for biosynthetic activity. Degradation of cellular components throughout the sporangiophore may be necessary since no substrate hyphae were present to supply it with the required substrates.

The accumulation of nuclei and mitochondria during growth resumption was also determined using the staining

methods and a greater intensity of staining was found at apical region, see Plates (8(4),15(2)). No differences observable by staining were found in the cytological distribution of RNA, DNA and protein during the initial growth of isolated sporangiophores, but differences in the quantities of macromolecules were found by extraction methods. This might be due to the fact that the staining methods were not sensitive enough to show slight variation in distribution.

Accumulation of wall staining intensity at the tip and at basal cut end of a cultured sporangiophore is shown in Plate (16(1,3)). A greater accumulation of wall stain appeared at the tapered tip of the sporangiophore than at the basal end. More intensity of staining for the wall (Uvitex BOPT) also can be seen at the tip of the hyphae regenerated from the basal end of the isolate sporangiophore, Plate (16(2)). Similarly a greater intensity of staining for RNA was found at the same region, Plate (47(3)). Thus there might be a correlation between RNA and wall substance synthesis during growth and development.

Regenerated hyphae were seen to have domed extreme apices, while in the same preparation other branched hyphae had tapered tips, Plate (16(2)). These

observations are in agreement with observations reported by Steel & Trinci (1975) for growth of hyphae of Neurospora crassa. They interpreted the dome-like tip as a characteristic of the main (undifferentiated) hypha which branched and which arose from the main hypha.

3. Distribution of cellular components during abnormal growth at the tip region

Some sporangiophores were shown to have abnormal features of growth by forming branches. More than one branch was seen to emerge from the sub-apical region of the sporangiophore, Plate (17). Growth of the branched sporangiophore was measured and the overall pattern of growth curves was found to be similar to those found for the isolated and attached sporangiophores.

Sporangiophores which showed branching were stained by Giemsa and by Korson's stains and are shown in Plate (17). It was found that the branching was preceded by changes in the location of the cellular components and the original apex of the sporangiophore appeared to be empty after branch formation, Plate (17(4)). Thus it is possible that the branching was associated with the inhibition of the normal apical growth. Altering the location and direction of movement of the cell wall precursors can be also considered as a reason leading to the impairing of the normal apical extension, since

Plate (18(2)) showed more intensity of wall staining (Uvitex BOPT) in the branched sporangiophore, while no stain was observed at the original tip. The location of the new regeneration and branching was seen to have greater intensity of wall stain. Thus the branching possibly involved a new synthesis of chitin. This suggestion resembles a suggestion made by Cabib et al., (1974), Cabib (1975), Cabib & Bowers (1975), since they observed that during the budding of yeast, the bud scar was shown to have a greater concentration of chitin. Therefore it has been suggested that the branching may be involved in activation of chitin synthase (see review by Gooday, 1978). Activation of chitin synthase of Phycomyces was found to occur by protease (Thomson & Fischer, 1976; Van Laere & Carlier, 1978; Fischer & Thomson, 1979). Liberation of protease during bud formation in the yeast was found by Hasilik (1974). Thus the location of the branched scar during regeneration and branching of the sporangiophore may correspond with the partial location activation of chitin synthase and thus wall synthesis. Liberation of substances or an enzyme(s) which are required for activating chitin synthase may be induced by mechanical injury.

Location of nuclei RNA and protein also changed and they were seen to accumulate in the branched sporangiophore, Plates (17(1,2,4), 19, 27(5), 29, 32)). The location of regeneration (branch scar) was also stained intensely for RNA, Plate (17(3)) and protein, Plate (19(1,2,3)). Thus the abnormal growth may also involve an activation of RNA and protein synthesis. An accumulation of stained nuclei at the site of the branch formation was also observed, Plate (19(4,5)). The formation of the branch appeared to be as a result of the localized accumulation of cellular components. Changing the normal distribution and organization of the cellular components appeared to impair the apical extension and thus activated the abnormal growth. Damage to the extreme apex was found to be associated with branching and each of these branches gave normal growth. Plates (15(1,2), 9(4)) show the accumulation of nuclei and mitochondria during injury of the extreme apex. Injury of the sub-apex was shown to activate the regeneration and branching. Thus it can be concluded that the intact sub-apical region is essentially required in maintaining the apical growth. This might be acceptable since this region was found to have a greater intensity of nuclei, mitochondria and a greater concentration of RNA, DNA, protein and chitin as found by Jan (1974). Additionally, greater intensity of wall staining



(Uvitex BOPT) was seen in the branched sporangiophore after regeneration, Plate (20(1,2,3,6)).

11. INTER-ASSOCIATION BETWEEN THE CONTINUITY OF THE  
CELLULAR COMPONENTS AND APICAL GROWTH OF  
SPORANGIOPHORES

The essential requirements for the continuity of the cellular components and the maintenance of apical growth of the sporangiophores were tested by ligating the tip region of the isolated sporangiophore with a very fine thread, this led to a break in the coenocytic continuity of the cytoplasm along the sporangiophore. The manipulated sporangiophores were cultured on solid minimal medium and were stained with Korson's stain for protein, RNA and DNA.

At 4-6 hours after the manipulation the extreme apex showed branching, see Plate (20(5)). More than one branch was formed side by side and they showed a greater intensity of the stain. The basal end of the tip segment, i.e. above the ligation, showed less intensity of the stain; increasing the internal pressure of the protoplasm as a result of the ligating could cause a change in the uniform synthesis of the wall substances and thus may activate branching. Likewise the internal damage of the protoplasm during the manipulation could also lead to abnormal growth.

Attached sporangiophores were also manipulated and ligated in a similar way as described above for the isolated sporangiophore. At 4-6 hours after ligating,

segment above ligation showed it had resumed growth, see Plate (20(7)). Greater intensity of stain for RNA and DNA seen at the basal segment below the ligation of the attached sporangiophore, compared with that seen at the basal segment below the ligation of the isolated sporangiophore, Plate (20(5)). Additionally the apical end of the basal segment i.e. below the ligation of the attached sporangiophore appeared more erect than that of the apical end of the basal segment of the isolated sporangiophore, Plate (20(5,6)). A continuous supplement of the cellular components from the mycelium into the basal segment below the ligation of the attached sporangiophore could maintain the internal pressure of the attached sporangiophore. The autolysis of cellular component at the basal segment below the ligation of the isolated sporangiophore could be correlated with less intensity of stain of RNA and DNA.

After ligating the isolated sporangiophore and cutting of the apex region, protein staining intensity was shown greater at the point of wound healing and regeneration i.e. at the cut level, Plate (20(4)). The basal end of the tip segment i.e. above the ligation, appeared translucent (no accumulation of protein was found). The apical end of the basal segment i.e. below the

ligation, of the attached sporangiophore showed a cylindrical shape and also developed a sporangiophore, Plate (14( 6)), while no development was seen at the apical end of the isolated sporangiophore. Additionally this could be also attributed to the internal pressure of protoplasm of attached sporangiophore which may be maintained by a continuous supplement of cellular components from the mycelium into the attached sporangiophore.

The basal end of the basal segment i.e. below the ligation, also showed wound healing and showed greater intensity of protein than the apical end of the basal segment. This could be attributed to the ligation effect, likewise no development of a sporangiophore was seen. In contrast there was a development of a sporangiophore at the apical end of the tip segment i.e. above the ligation.

Accumulation of protein at the apical end of the tip segment can be used as evidence for the quicker redistribution of cellular components and thus resumption of protoplasmic streaming, from the basal end to the upper end. Therefore quicker recovery and regeneration at the upper end of the tip segment may be attributed to the redistribution of cellular components.

Likewise it is possible that the maintenance of apical growth extension of the normal sporangiophore can

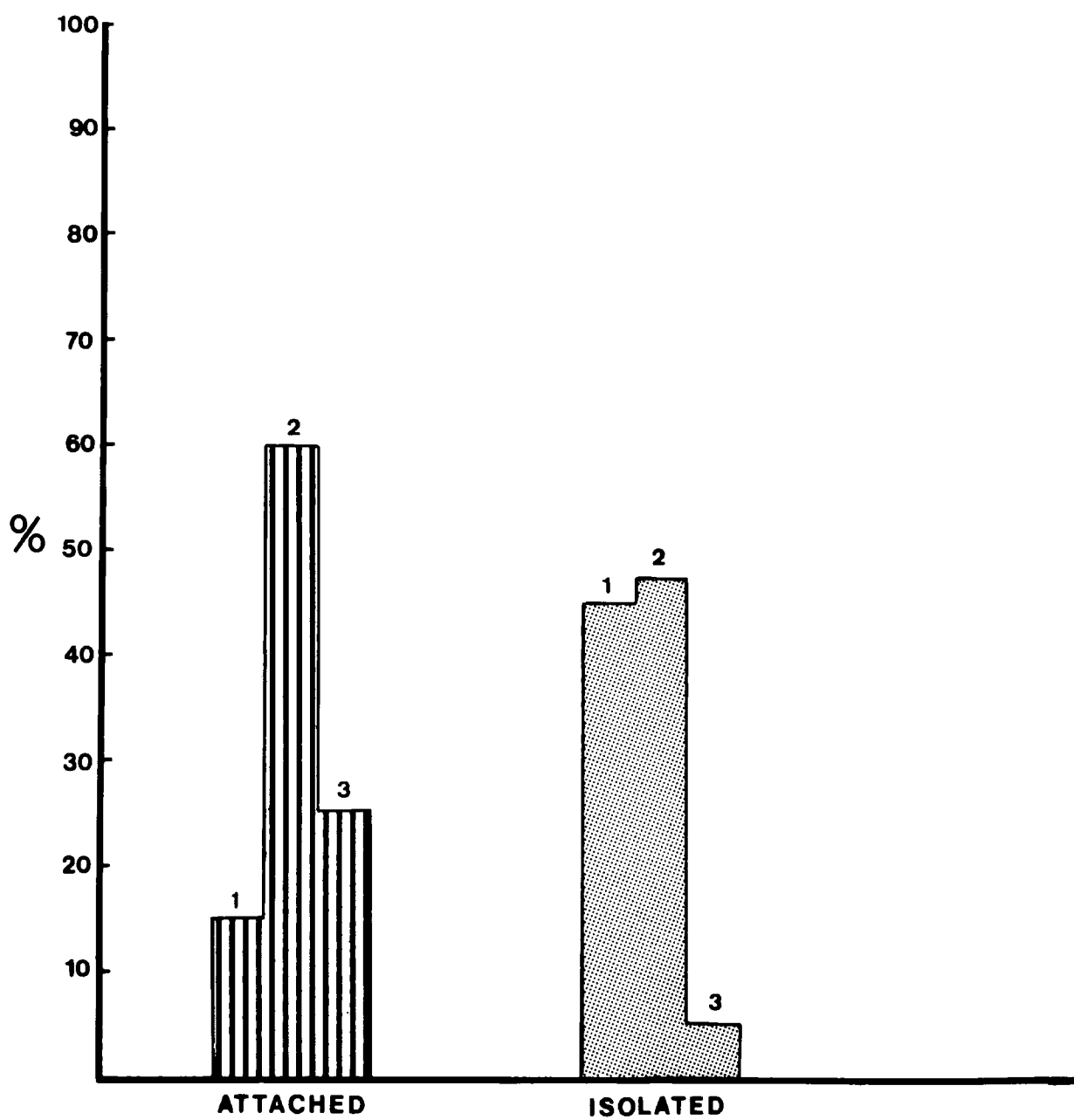
occur at the expense of other compartments below the apex region. These observations also support the suggestion of the possibility of degradation or autolysis of cellular components in parts below the tip region which can then be utilized at a location where components are required for growth. Also movement without autolysis is possible.

A comparison between the regeneration of the stump on an attached sporangiophore and of a comparable isolated segment of sporangiophore was also made. In the former case, the sporangiophore was excised to leave a 15 mm stump and a comparable length of a detached sporangiophore was also used. The amount of the regeneration is summarized in Figure (14). Regenerating sporangiophores developed both from the centre and the peripheral of the original sporangiophore, sometimes one, sometimes two and rarely three sporangiophores developed. New sporangiophores were produced from the isolated segments also, but a higher proportion of the attached segment gave more than one sporangiophore than did the segments. More intensity of stain of Uvitex BOPT stain was shown in the attached condition at the stump compared with the isolated sporangiophore. This indicates that the contribution of the mycelium is important in sporangiophore regeneration, possibly through a supply of growth components. This was

Figure (14)

Percentage of regeneration of sporangiophores at cut ends of stumps on attached sporangiophores and of comparable isolated sporangiophores. The isolated sporangiophores were cultured on the solid minimal medium.

1. One regenerated sporangiophore
2. Two regenerated sporangiophore
3. Three regenerated sporangiophore



found when the stump on an attached sporangiophore with cut apex region and of a comparable isolated segment of sporangiophore, were stained for RNA, DNA and protein during regeneration, Plate (21). Greater intensity of RNA, and DNA was found (at 6 hours) at the apical end of the stump on the attached sporangiophore, Plate (21(2)), compared with those found at the apical end of the same stump before regeneration, Plate (21(1)). The comparable isolated segment showed less intensity of stain for RNA and DNA at the apical end of isolated sporangiophores, Plate (21(3)). The basal regions of stumps showed a greater intensity of stain for RNA and DNA compared with the isolated segment, Plate (21(2,3)). Thus the continuous supplement of cellular components from the mycelium into the stump could be contributing to this increase of nucleic acids and thus a greater recovery. In contrast few nuclei were seen at the basal region of the isolated sporangiophore, Plate (21(4)). Less intensity of staining for DNA and RNA and greater intensity of protein at the part below the apical end of the isolated sporangiophore could be attributed here to the autolysis. Some autolysis or degradation can also be seen at the middle region of the stumps especially that shown in Plate (21(1)). This could be attributed to the extension of the mechanical effect.



Less autolysis or degradation at the middle region of the stump, shown in Plate (21(2)), could be interpreted as the middle region of that stump receiving a continuous supplement of cellular components from the obconical region of the attached stump. Recovery then may take place at the expense of the attached basal region of the stumps, Plate (22(1)).

Plates (22(2), 23)), show the distribution of the nuclei at the basal region of the isolated sporangiophore, where no accumulation of cellular components and nuclei can be seen at the obconical region. In contrast greater nuclei were found at the basal region of the stump, Plate (23(1,2)), compared with the comparable isolated sporangiophore, Plate (23(3)). Thus a continuous supplement of nuclei from the substrate-hyphae into the stump can be used as evidence for the greater accumulation of nucleic acids throughout the attached sporangiophore. The accumulation of wall substances at the ends of the stump, also can be attributed to a greater activity of wall synthesis and thus regeneration, Plate (33(4)).

12. INTER-ASSOCIATION BETWEEN RNA, NUCLEI AND VESICLES  
DURING THE REGENERATION OF THE SPORANGIOPHORES

Nuclei and RNA were determined by the acridine orange stain, during the regeneration and growth of the sporangiophore which had a cut extreme apical region, Plate (24). Vesicles like structures were also seen in the preparation, and these were found to be more abundant at the inner site of the regenerating zone and of the original wall, Plate (24(1)). Some vesicles were found surrounded by nuclei. Therefore, it is possible that there is an inter-association between the nuclei and the formation of vesicles. The possible inter-association between the nuclear envelope and the formation of the vesicles of fungi has been reviewed by Weber & Hess (1976). Likewise the regeneration of the new wall appeared to require the presence of the vesicles.

A continuous supply of the enzyme-containing vesicles including the wall building blocks, from the lower compartments of the fungal hyphae towards the apical parts of the hyphae was assumed by Trinci (1974). After the wall closure at the injured area, nuclei and RNA appeared to accumulate at the apex and continued to be associated with the extension of the apical region, Plate (23(2)). Nuclei found in the sub-apical zone, Plate (23(3)), and at the regenerated regions were more

dense and slightly bigger than those found at the basal end of the sporangiophore, Plate (23(4)). In addition few and indistinct vesicles and nuclei were seen in the lower regions of the sporangiophore. RNA staining was also found in less intensity at the end of the basal region. More intensity of RNA staining and a greater number of nuclei and vesicles like structures were found at the apical end of the tip region of the sporangiophore during wall synthesis and apical extension.

The essential requirement of nuclei in wound healing was also tested by incision at the middle region of the sporangiophore with a needle and then the sporangiophore was stained for nuclei with Feulgen stain. Plate (25(2)) shows the accumulation of the nuclei around the injury. Such an accumulation could be due to the selective transport, to the site of injury of substances involved in the healing process. So nuclei could play an important role during wall synthesis and growth of the sporangiophore.

13. EFFECT OF THE CUTTING INTO SEGMENTS ON THE DIFFERENTIATION AND MORPHOGENESIS OF SPORANGIOPHORES

Stage I sporangiophores lengths 15-20 mm were cut into 3 to 5 segments of equal length in each experimental set. Methods used for cutting are described in materials and methods. By such manipulation the location of the region with greatest ability to regenerate was sought.

Growth curves are shown in Figure (15). Most of the segments reached the final stage (IV). The main point of interest was that there was a gradation of regeneration rate down the segment sequence from the apex. The apical segment regenerated most quickly to stage IV whereas the basal segment was slowest. However, this time difference may be partly accounted for by the fact that the length of the regenerated sporangiophore was greater at parts below the apex segments.

The location and numbers of the regenerated sporangiophores at the cut sites of the tip, middle and base segments are shown in Figure (16). It can be seen that a greater number of regenerated sporangiophores were found at the apical cut end of the tip segment. Thus the regeneration and branching may be activated by mechanical injury.

Figure (15)

Growth curves of sporangiophores developed from apical ends of segments:

- Tip segments with lacking apex region
- \* Tip segments with completed apex region
- △ Middle segments
- ▲ Sub-middle segments
- Basal segments

Each value is a mean of 3 replicates. The segments were equal in length (3-5 mm) and were cut from synchronized sporangiophores which were isolated from the same culture. Segments were cultured on the solid minimal medium.

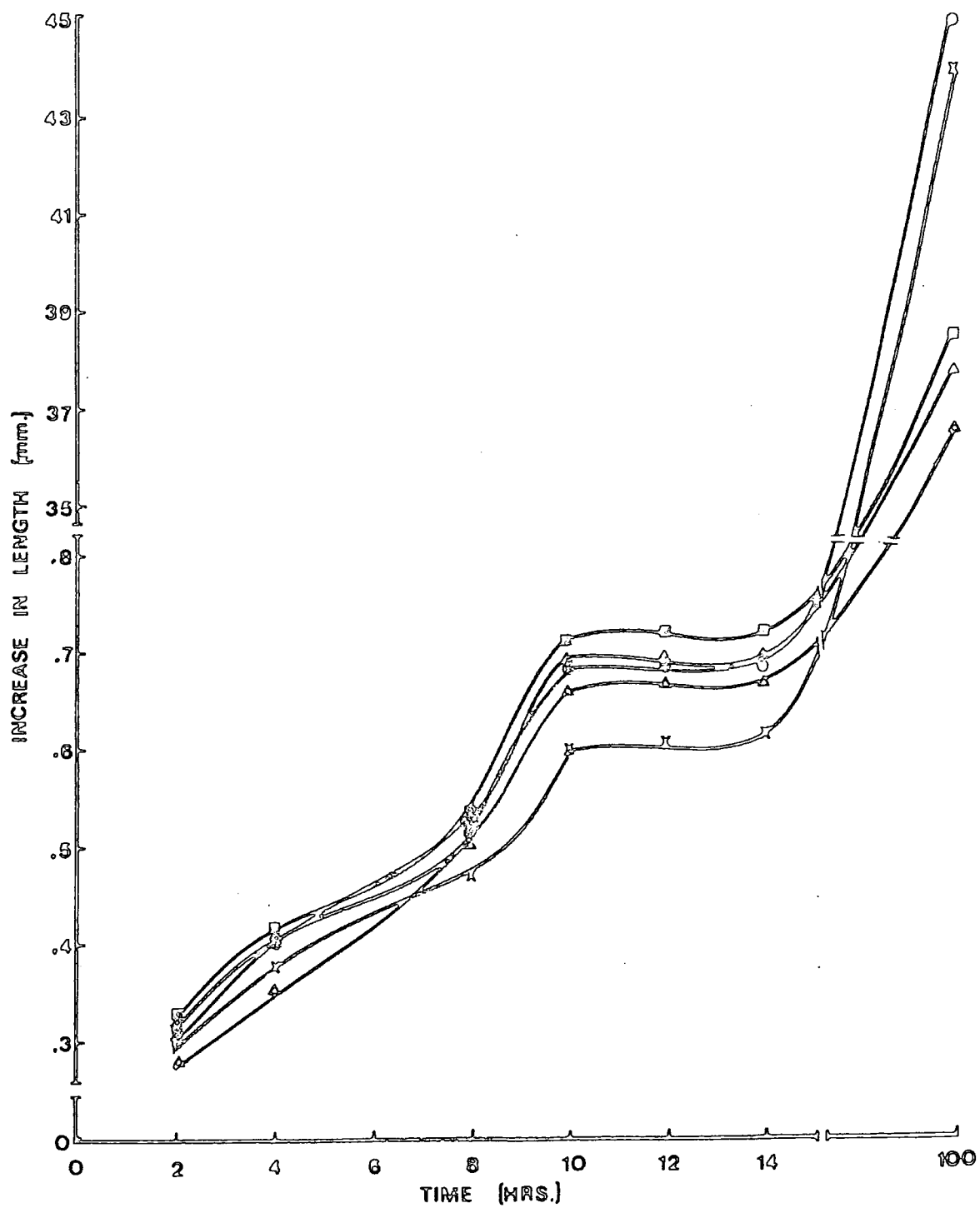


Figure (16)

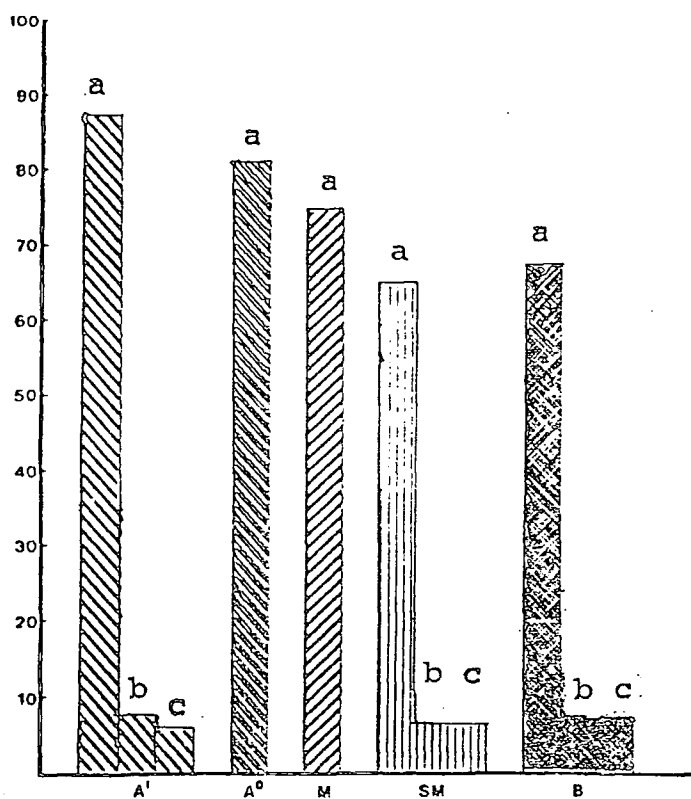
1. Percentage of location of regeneration at the apical cut ends of segments

- a - indicates apical growth
- b - indicates lateral or branched growth
- c - indicates both

2. Percentage of numbers of developed sporangiophores from the apical cut ends of segments

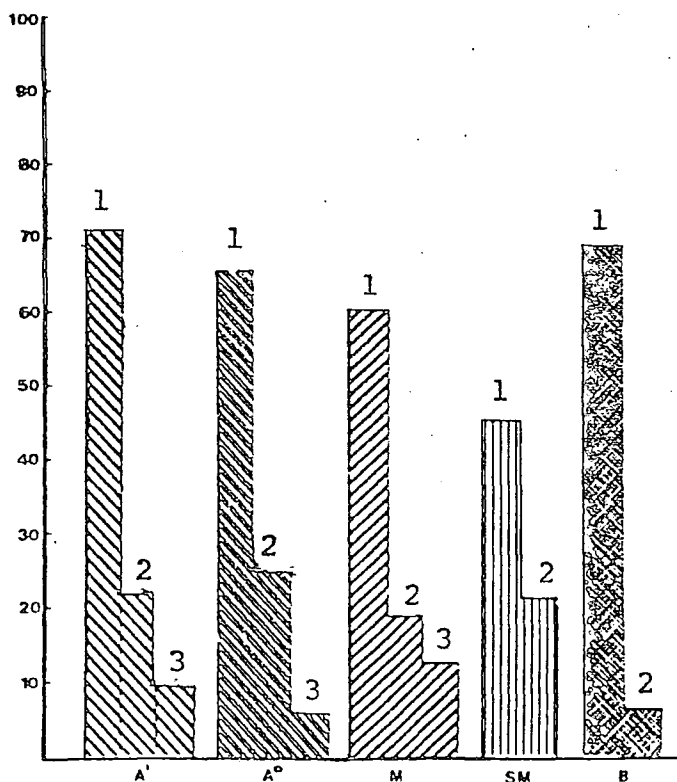
- 1 - indicates one regenerated sporangiophore
- 2 - indicates two regenerated sporangiophore
- 3 - indicates three regenerated sporangiophore

Percentage of location  
of regeneration



Segments

Percentage of numbers of  
developed spms.



Segments



The regeneration and development of sporangiophores which were cut into 5 segments (A,  $\overset{\circ}{A}$ , M, SM and B) is shown in Plate (26). A, represents the tip segment with an extreme apex intact, while  $\overset{\circ}{A}$  represents the tip region lacking an extreme apex. M and SM represent the middle and the sub-middle, while B represents the basal regions.

The location and number of regenerated sporangiophores at the cut ends of each segment are summarised in Figure (14). More regeneration was found at the tip segment which were lacking an extreme apex. More than two regenerated sporangiophores were found to emerge from the lateral site of the  $\overset{\circ}{A}$  segment.

The A segment showed no branching and continued growth as an attached sporangiophore. Resumption of normal accumulation of nuclei, could have contributed to this growth, Plate (27(3)). Occasionally branches were seen arising from the sub-apical region, Plate (26(A)), possibly as a result of damage in this region.

Branching was also seen arising from the sub-apical region of the tip segment with cut extreme apex, Plate (26(A)). Thus inhibition of the apical growth may stimulate sub-apical growth and sub-apical branch formation.

Where sporangiophores regenerated from the cut ends of segments other than apical segment with its extreme apex intact, they arose from lateral sites. Therefore the tip appears to control the site of origin of the new sporangiophore. On excision, it was found that there was no re-establishment of the original tapered tip but a new sporangiophore with a tip produced from internal sites in a lateral position was seen. This may be related to the location of cytoplasm at the peripheral, surrounding the vacuole zone; which acted as a regeneration site, see Plate (28(5)).

These observations are an indication of polarity in that the regeneration at the apical end of each segment gives rise to a new sporangiophore. Likewise this could be correlated with the non-uniform distribution and formation of cellular components along the segment length. Additionally more regeneration was found at the sub-apical segment and this may be correlated with the presence of more cellular components since the dry weight of the tip segments were seen to increase in the dry weight during regeneration, Figure (17).

1. Distribution of RNA, protein and DNA during regeneration and growth of sporangiophore segments

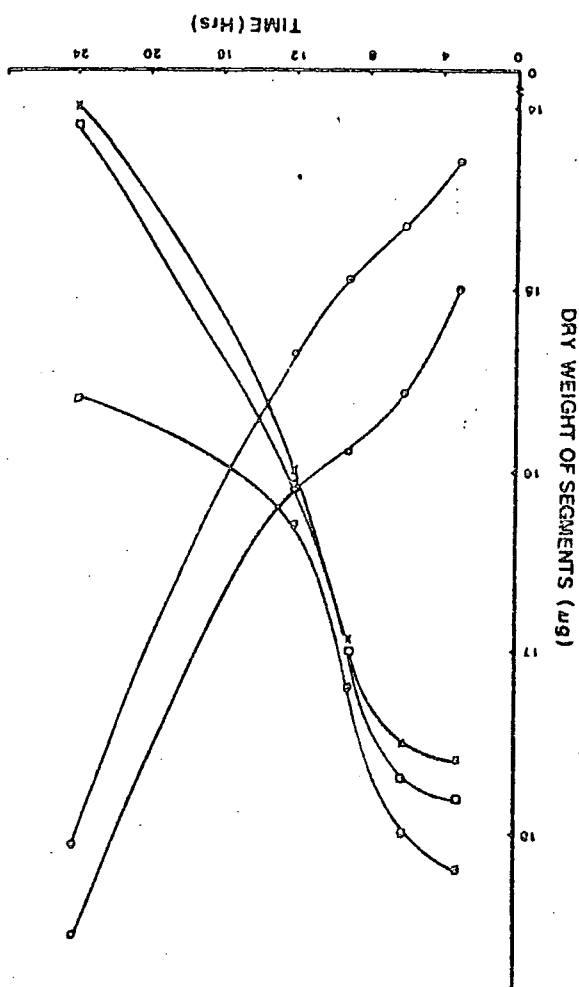
Total concentrations of RNA, protein and DNA were extracted and estimated at time intervals during

Figure (17)

Dry weight measurements during regeneration of segments after regeneration and development of sporangiophores.

- Tip segment with intact apex region
- Tip segment lacking apex region
- Middle segment
- Sub-middle segment
- ◇ Basal segment

Known numbers of segments were weighed at intervals during regeneration and development; the weight of each individual segment was then calculated



regeneration and growth of segments (tip, middle and base) of sporangiophores. Changes in the quantities of these macromolecules are shown in Figure (18).

As shown, during the initial hours of incubation there was a decrease in the concentration of RNA and DNA, but there was an increase in the concentration of the protein. Mechanical manipulation may have caused a disruption of the protoplasm which may have brought about an imbalanced condition within the cell, as suggested by Fenc1 (1978). This could have contributed to the initial decrease of the RNA and DNA. A possible explanation for the initial increase of the protein, is that the cutting may have led to a synthesis and release of some enzyme(s) involved in repair of wounding. The tip segment showed more initial increase in the protein concentration than did the middle and basal segments. This could be attributed to the fact that the tip region contains more membrane-bound organelles (Peat & Banbury, 1967; Marchant et al., 1967; Thornton, 1968a; Bergman et al., 1969; Tu et al., 1971; Hankinson, 1972) and thus may produce more abnormal release of protein.

The normal recovery was achieved by a decrease of the abnormal rise of the protein and following this there was then a gradual recovery of RNA and DNA levels. Comparison

Figure (18)

Concentration of macromolecules extracted at different intervals of regeneration and development of segments of stage I sporangiophores.

1.

Tip

☒ protein

☐ RNA

☐ DNA

2.

Middle

☒ protein

☐ RNA

☐ DNA

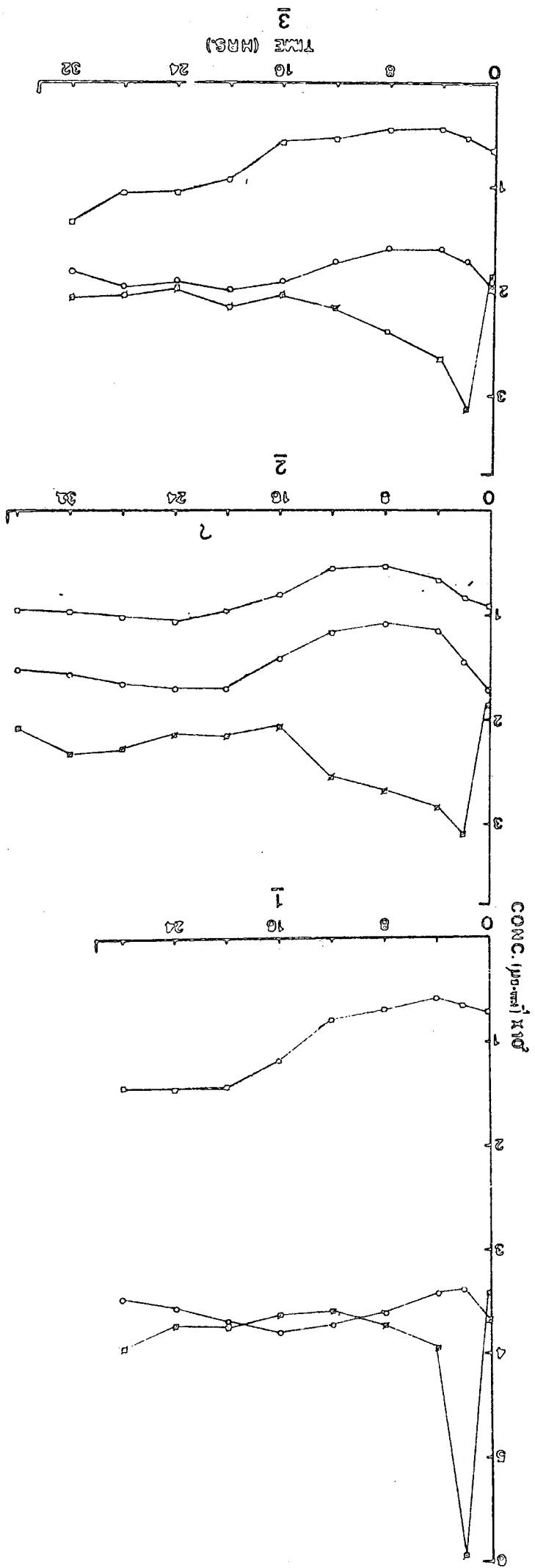
3.

Base

☒ protein

☐ RNA

☐ DNA



with the control (RNA, DNA and protein were extracted immediately after cutting and freezing of the tip, middle and basal segments) showed that the maximal recovery of these macromolecules were found at 6-8, 10-12 and 14-16 hours, for the tip, middle and basal segments respectively. These time sequences correlated with the times of the initiation of regeneration.

The location of RNA, DNA and protein was also determined cytologically, and Plates (27, 28, & 29) show their distribution only in tip segments. Accumulation of these components took place at the site of new wall synthesis. The recovery of the RNA and DNA at the cut site appears to be at the expense of the other parts of the segment. The accumulation of these macromolecules was also observed to be associated with accumulation of nuclei thus accounting for the nucleic acid synthesis, Plate (28(2)). Therefore, it was concluded that the total concentration recovery of the components measured was the same for the tip, middle and basal segments, but within each segment differences of distribution were seen.

## 2. Distribution of nuclei during regeneration

The tip segment with a lack of the apex region was shown to have abnormal feature branching of growth as shown in Plate (30(1)). A branch was formed at 4 hours



after cutting and was seen to be associated with changes in the normal distribution of the nuclei. The site of branch formation appeared to have a greater number of nuclei than the region which was damaged internally, Plate (30(2)). Such damage could be attributed to the effect of the mechanical cutting. Thus changing the normal distribution of nuclei could produce an abnormal growth. Changing by mechanical shock the normal distribution of organelles involved in carrying the wall precursors could be considered to such abnormal branch formation.

Usually nuclei were seen to accumulate at the cutting site of segments and this accumulation was followed by wound healing and then sporangiophore developed from the regenerated site. As shown in Plate (30(3,4)), more accumulation of nuclei can be seen at the apical end of the tip segment, where the branched sporangiophore developed.

In some cases as shown in Plate (31(1)), no branch formation can be seen but the dome like tip structure was developed after wall healing or septum formation. A greater accumulation of nuclei can be seen in the area producing the septum. Using high magnification of the preparation as shown in Plate (31(2)), there was a

difference in the size and intensity of staining for nuclei. Nuclei which are below the regeneration show greater intensity of stain (Feulgen) and a smaller size compared with those above the regeneration. Nuclei above the regeneration site appeared larger and also showed what appeared to be fragmentation into smaller nuclei or more distinct chromosomes. Normal mitotic division was not seen. Such fragmentation of nuclei can be attributed here to the possible proliferation of the nuclear envelope and such an explanation was considered because of the abnormal condition of the sporangiophore. Since an inter-association between nuclear envelope and vesicles formation has been found (see Weber & Hess, 1976) it can be suggested that the proliferation of fragmentation of nuclear envelope could be related to the formation of cell wall precursors. Since similar preparations were stained for wall synthesis using Uvitex BOPT and these showed a greater intensity of stain bound to the wall, at the region which showed fragmented nuclei. The intensity of stain binding was less at parts below the regeneration, Plate (31(3,4)). Additionally, an unknown dense body which stained intensely with Feulgen stain, was seen below the regeneration site, and it is suggested that it may have a function in directing growth. Such a body may resemble in its function the 'spitzenkörper' body

which was found at the extreme apex of the hyphae of fungi such as Neurospora crassa and Rhizoctonia solani (see Girbardt, 1957 and reviewed by Grove, 1978). Additionally, since it stained intensely by the nuclear stain, it seemed to be correlated with the nuclei location and direction.

Tip segments with lacking apices also showed the development of more than one branch as shown in Plate (32(2)). Nuclei were also seen distributed along the regenerated sporangiophore and stained intensely with acridine orange. Less nuclei were seen accumulated at an apical end of the middle segment as shown in Plate (32(3)). The regeneration site or septum was also stained intensely by Feulgen stain.

After regeneration of the basal segment, it was stained with Feulgen and shown in Plate (32(4)). Nuclei can be seen distributed less densely along the developed sporangiophore. A dense body can also be seen at the basal region of the developed sporangiophore. No septum was seen. Thus, such a body was seen in more than one preparation at the basal region of the regenerated and developed sporangiophores. Thus it may reflect a significant physiological state of the growth direction. Such a body was not found during normal growth of the sporangiophore and thus it can be suggested that it is

not apparent during normal growth but reappears in some cases of abnormal growth of regeneration and branching.

Generally, distribution and accumulation of nuclei appeared to vary from segment to segment and greater accumulation was found at the tip region of sporangiophore which may be correlated with more metabolic activity and apical growth extension.

### 3. Distribution of wall substances during the regeneration and development of sporangiophores

The location of the wall polysaccharide fractions was determined cytologically, and quantitatively estimated using Uvitex BOPT stain, by the methods described in materials and methods. The ability of the fluorescent stain to bind to the polysaccharide fraction was utilized to give information about the location and contents of the cell wall produced during sporangiophore regeneration.

Synchronized sporangiophores of stage I were cut into a series of 3 to 5 segments. They were cultured on solid minimal medium following a method described in materials and methods. During regeneration, the concentration of adsorbed stain per section was estimated as shown in Figure (19). The amount of polysaccharide accumulated may thus reflect the activity of wall synthesis.

Figure (19)

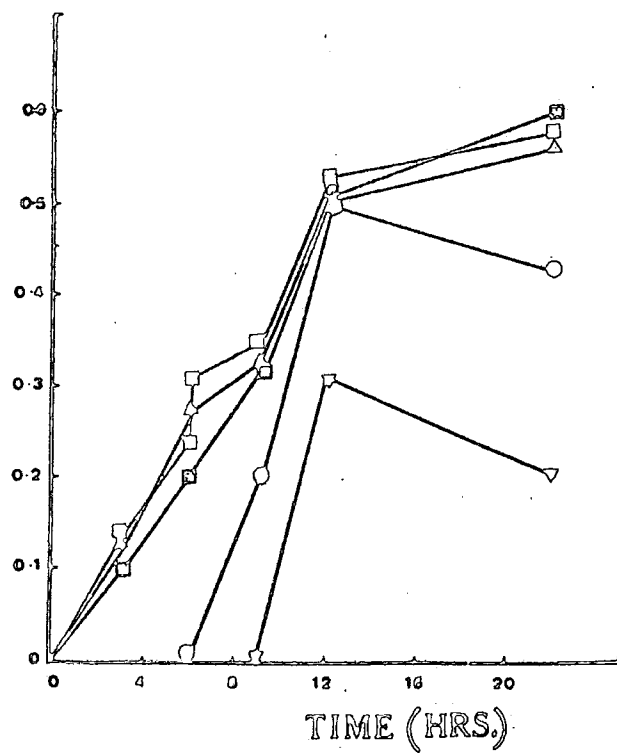
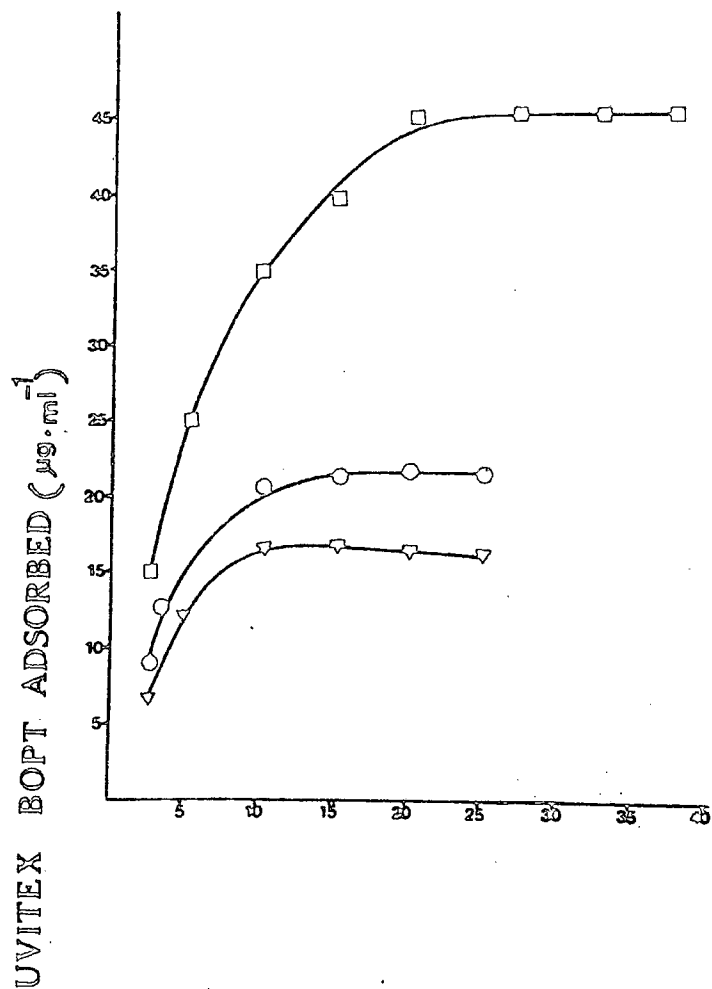
The amount of Uvitex BOPT stain adsorbed by walls of segments during regeneration and development. 1 mg dry weight wall per 5 ml standard stain solution was incubated in the dark for 4 minutes. The amount of stain adsorbed by the wall was calculated from the calibration curve after measuring the absorbance at 350 nm (see materials and methods of Chapter I).

1. Sporangiphores were cut into 3 equal length segments (5 mm)

- Tip segments
- Middle segments
- ▽ Base segments

2. Sporangiphores were cut into 5 equal length segments (5 mm)

- Tip segments
- ▣ Tip segment but with no apex regions
- △ Middle segments
- Sub-middle segments
- ▽ Base segments



The highest concentration of stain was found in the tip segment with the least in the basal segment. Adsorption of stain started at 4-5 hours i.e. before regeneration started. Maximal adsorption was found after approximately 10 hours for base, 14 hours for middle and 16 hours for tip segments. The apical end of tip segment showed a greater intensity of Uvitex BOPT than other segments. This could be related to the degree of regeneration and indicates areas of high wall synthetic activity, Plate (33).

As shown, the area which exhibited greater intensity of wall staining, also showed a greater number of nuclei and more concentration of nucleic acids and protein, further indicating high metabolic activity.

14. EFFECT OF NUCLEIC ACID AND PROTEIN SYNTHESIS INHIBITORS, AND NUCLEAR DIVISION INHIBITOR ON GROWTH AND DEVELOPMENT OF SPORANGIOPHORES

The role of nucleic acid and protein synthesis in the growth and development of sporangiophores was tested by incubating sporangiophores of stage I in actinomycin D (as an inhibitor of RNA synthesis at the transcriptional site), 6 - methylpurine (an RNA analogue inhibitor), rifampicin (an inhibitor of organelle RNA Polymerases), ethidium bromide (an inhibitor of DNA synthesis of mitochondria), cycloheximide (a potent inhibitor of protein synthesis on cytoplasmic ribosomes), MDMP (2-(4-methyl-2, 6-dinitro anilino) -N-methyl proprionamide) inhibitor of protein synthesis, and colchicine, an inhibitor of mitotic division of nuclei .

The minimal effective concentration of each inhibitor was determined, by incubating sporangiophores on solutions of 10, 20, 30, 40, 50, 60, 80, 100 and 200  $\mu\text{g}.\text{ml}^{-1}$  in distilled water. Sporangiophores which were cultured in water were used as controls. Sporangiophores which were manipulated were isolated from the culture and used for the entire series of experiments. The basal ends of isolated sporangiophores were carefully dipped in the inhibitor solutions following the same method previously described for culturing isolated



sporangiophores. The length increase of the sporangiophores were measured using a travelling microscope. Table (3) shows the mean values of growth against the concentration of the inhibitors. Effective concentrations used in the experimental sets were as follows:-

|                  |                                 |
|------------------|---------------------------------|
| Cycloheximide    | 20 $\mu\text{g}.\text{ml}^{-1}$ |
| 6-Methylpurine   | 25 $\mu\text{g}.\text{ml}^{-1}$ |
| Actinomycin-D    | 25 $\mu\text{g}.\text{ml}^{-1}$ |
| Rifampicin       | 25 $\mu\text{g}.\text{ml}^{-1}$ |
| Ethidium bromide | 25 $\mu\text{g}.\text{ml}^{-1}$ |
| MDMP             | 25 $\mu\text{g}.\text{ml}^{-1}$ |
| Colchicine       | 25 $\mu\text{g}.\text{ml}^{-1}$ |

Of the inhibitors used it was found that only cycloheximide and 6-methylpurine inhibited growth of the sporangiophore to stage IV. However, the final length of sporangiophore was reduced by all the inhibitors. Higher dosages of the actinomycin-D, rifampicin, ethidium bromide, MDMP and colchicine ( $200 \mu\text{g}.\text{ml}^{-1}$ ) were found to inhibit the growth of sporangiophores, this might be due to the concentration blocking the whole synthetic machinery of the sporangiophore, since higher dosages of actinomycin-D was found not only to inhibit mRNA synthesis but also the whole synthetic machinery of the cell (Yoshino et al., 1968; Fagard & Saadi, 1977, cited by Ramadani, 1978).

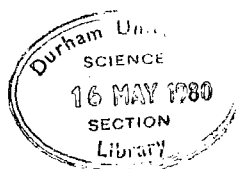


TABLE (3)

The effect of nucleic acids, protein, synthesis and nuclei division inhibitors, on the growth and development of sporangioophores (1.5 cm in initial length). The final length of the developmental stage is a mean of 3 replicates:-

| Inhibitors       | Concentration ( $\mu\text{g}\cdot\text{ml}^{-1}$ ) | Final developmental Stage | Final length (cm) |
|------------------|--|---------------------------|-------------------|
| Ethidium Bromide | 10   | IV                        | 7.4.              |
|                  | 20   |                           | 5.2               |
|                  | 30   |                           | 6.3.              |
|                  | 40   |                           | 6.8               |
|                  | 50   |                           | 6.6               |
|                  | 60   |                           | 6.0               |
|                  | 80   |                           | 5.4               |
|                  | 100  |                           | 4.0               |
|                  | 200  |                           | 3.8               |
| Actinomycin D    | 10   | IV                        | 7.1.              |
|                  | 20   |                           | 5.6.              |
|                  | 30   |                           | 5.5.              |
|                  | 40   |                           | 5.2               |
|                  | 50   |                           | 5.0               |
|                  | 60   |                           | 4.9               |
|                  | 80   |                           | 4.7               |
|                  | 100  |                           | 4.1               |
|                  | 200  |                           | 3.5               |
| Rifampicin       | 10   | IV                        | 7.3.              |
|                  | 20   |                           | 6.4.              |
|                  | 30   |                           | 6.1.              |
|                  | 40   |                           | 6.0               |
|                  | 50   |                           | 5.8               |
|                  | 60   |                           | 5.4               |
|                  | 80   |                           | 5.1               |
|                  | 100  |                           | 4.3               |
|                  | 200  |                           | 4.0               |
| 6-Methylpurine   | 10   | I                         | 2.0               |
|                  | 20   |                           | 2.0               |
|                  | 30   |                           | 1.9               |
|                  | 40   |                           | 1.9               |
|                  | 50   |                           | 1.8               |
|                  | 60   |                           | 1.8               |
|                  | 80   |                           | 1.8               |
|                  | 100  |                           | 1.5               |
|                  | 200  |                           | 1.3               |
| Cycloheximide    | 10   | I                         | 1.9               |
|                  | 20   |                           | 1.8               |
|                  | 30   |                           | 1.8               |
|                  | 40   |                           | 1.7               |
|                  | 50   |                           | 1.7               |
|                  | 60   |                           | 1.5               |
|                  | 80   |                           | 1.5               |
|                  | 100  |                           | 1.3               |
|                  | 200  |                           | 1.3               |
| MDMP             | 10   | IV                        | 7.2.              |
|                  | 20   |                           | 7.6.              |
|                  | 30   |                           | 7.2               |
|                  | 40   |                           | 6.7               |
|                  | 50   |                           | 6.6.              |
|                  | 60   |                           | 6.4               |
|                  | 80   |                           | 6.3               |
|                  | 100  |                           | 5.1               |
|                  | 200  |                           | 3.2               |
| olchicine        | 10   | IV                        | 6.9               |
|                  | 20   |                           | 6.5               |
|                  | 30   |                           | 6.7               |
|                  | 40   |                           | 6.4               |
|                  | 50   |                           | 6.4               |
|                  | 60   |                           | 6.4               |
|                  | 80   |                           | 6.0               |
|                  | 100  |                           | 5.9               |
|                  | 200  |                           | 4.1               |
| ontrol ( water ) |  | IV                        | 8.4               |

Sporangiophores treated with  $25 \mu\text{g}.\text{ml}^{-1}$  of actinomycin-D, 6-methylpurine, rifampicin, ethidium bromide, MDMP, colchicine and in  $20 \mu\text{g}.\text{ml}^{-1}$  of cycloheximide were used for the extraction of RNA, DNA and protein. The extraction was carried out at a time when sporangiophores reached a final stage of development (IV) under the control conditions. The results are shown in Table (4).

It was found that the growth inhibition of sporangiophores with the cycloheximide (at  $20 \mu\text{g}.\text{ml}^{-1}$ ) and with 6-methylpurine (at  $25 \mu\text{g}.\text{ml}^{-1}$ ) was associated with a decreased concentration of protein and RNA compared with the control. The concentration of RNA, DNA and protein of sporangiophores treated with other inhibitors was seen to resemble those of the control, but with a slight decrease in the sporangiophore which had been treated with actinomycin-D, rifampicin and ethidium bromide. A slight decrease in the concentration of protein was also found in the sporangiophores which have been treated with MDMP.

Given the limitation of the use of the inhibitors, it appears that the sporangiophore growth required protein synthesis and new RNA synthesis, which bears out the results on the accumulation of nuclei at sites of high RNA and

TABLE (4)

Total concentration of DNA, protein and RNA  $\mu\text{g}.\text{ml}^{-1}$  following the treatment with the inhibitors. Weights of sporangiophores were taken into account during calculations.

| <u>Inhibitors</u> | <u>Final stage<br/>of<br/>Development</u> | <u>Concentration (<math>\mu\text{g}.\text{ml}^{-1}</math>)</u> |            |                |
|-------------------|---|--|------------|----------------|
|                   |   | <u>DNA</u>   | <u>RNA</u> | <u>Protein</u> |
| Control           | IV  | 130  | 280        | 380            |
| Cycloheximide     | I   | 138  | 290        | 162            |
| MDMP              | IV  | 128  | 259        | 290            |
| 6-Methylpurine    | I   | 141  | 120        | 147            |
| Actinomycin D     | IV  | 112  | 230        | 330            |
| Rifampicin        | IV  | 174  | 208        | 310            |
| Ethidium bromide  | IV  | 98   | 278        | 366            |
| Colchicine        | IV  | 102  | 275        | 360            |

protein concentration. Failure of actinomycin-D may have been related to penetration problems.

The failure of inhibition by colchicine of the development of sporangiophore could be attributed to the fact that there was no division of nuclei during growth of the sporangiophore. No mitotic division of nuclei was found along the sporangiophore during its growth by Flanagan (1970), Frank & Reau (1971). Alternatively the concentration of this inhibitor or indeed the inhibitor itself may not have been effective in this particular fungus.

Plate (1)

1. Experimental growth chamber

2. Culture chamber for isolated sporangiophore

A. Transport cylindrical jar with screw lid

B. Small petri dish

C. Slide

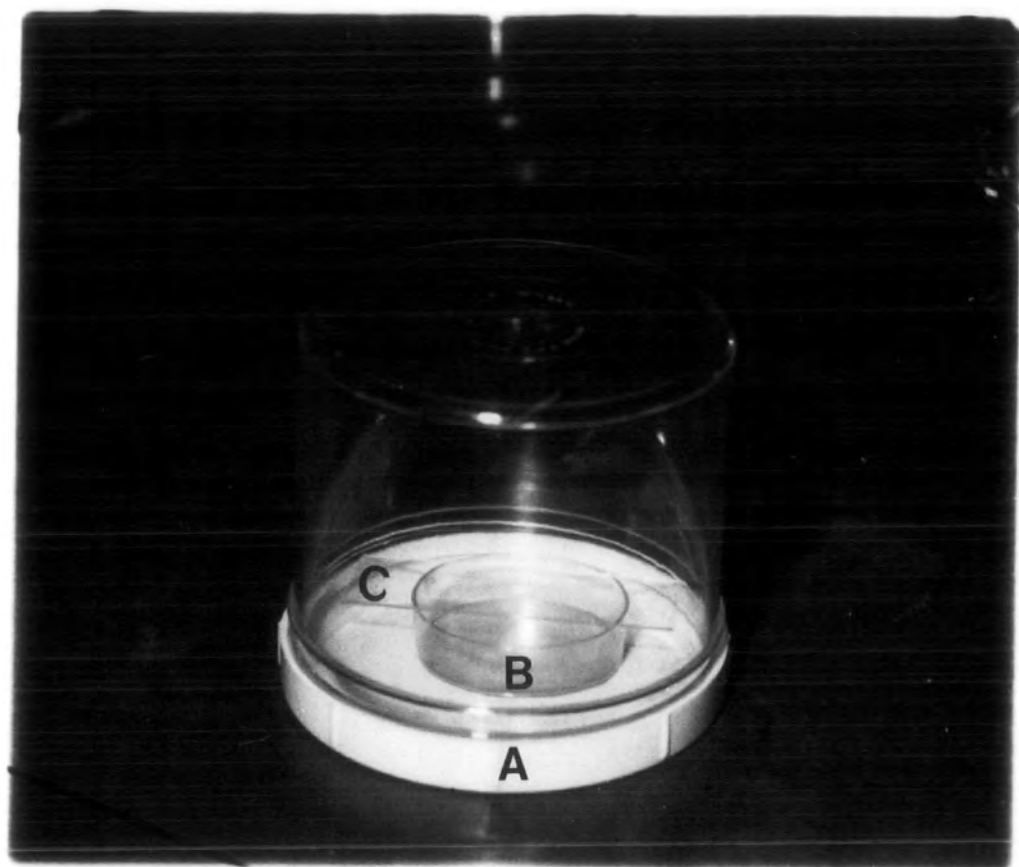


Plate (2)

Mycelia induced for synchronization

1. Zero hr : magnification X41.6
2. 2 hr : magnification X320
3. 3 hr : magnification X400
4. 4 hr : magnification X400

B: Branch

FC: Foot Cell

HC: Hypha like cylinder

T: Tip region of foot cell

The grown hyphae were fixed in situ by acetic acid alcohol (1:3 v/v) for 30 minutes, washed by water for 5 minutes and then stained by 0.1% solution toluidine blue for 5 minutes. The preparations were destained by ethylalcohol (100%) for 2 hours.



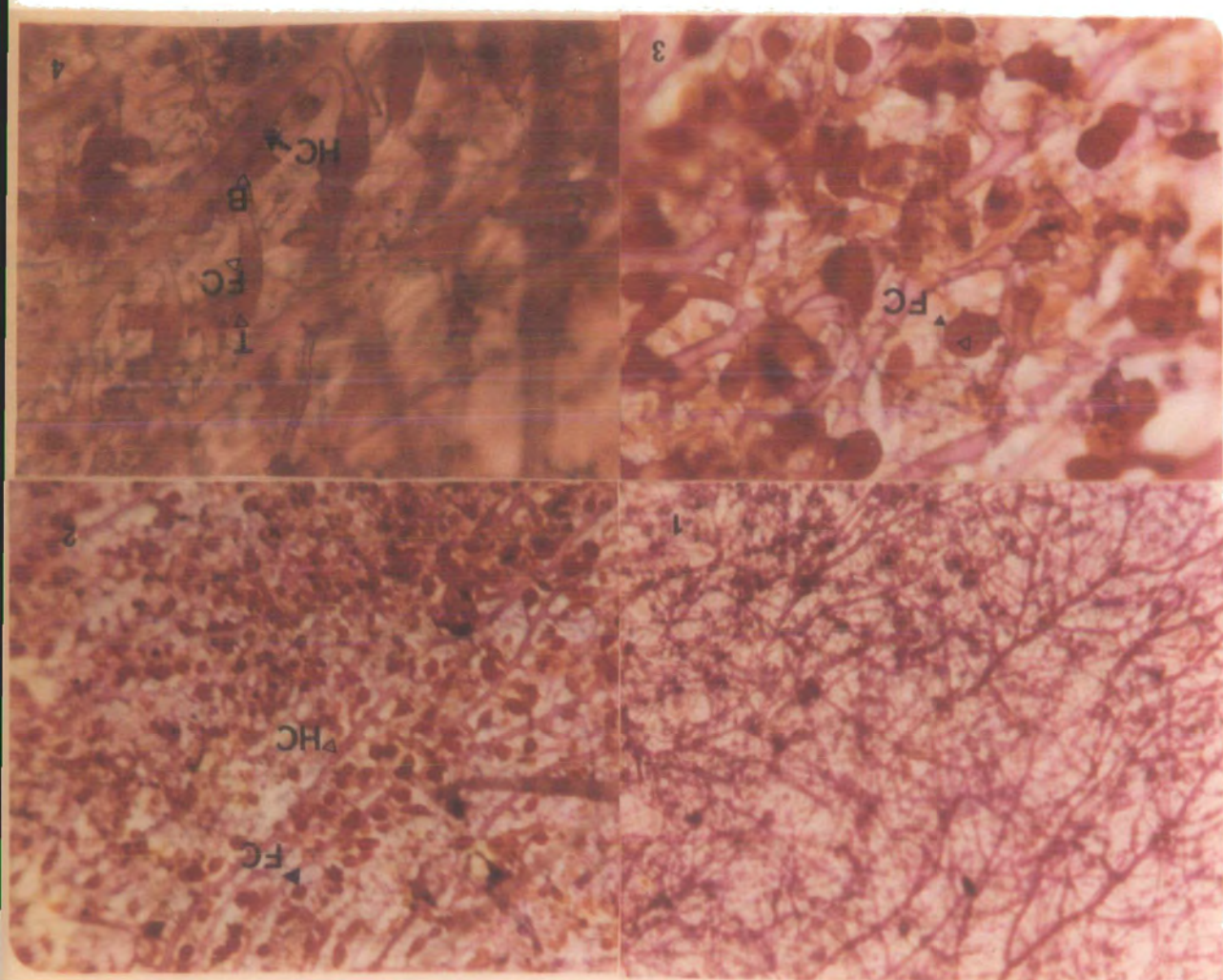


Plate (3)

1. Foot cells formed by non-synchronization in growing hyphae

magnification X400

2. Different zones or regions of maturation, during development of hyphae non-treated for synchronization production of sporangiophores.

- 1 - A region characterized to have tips leading to hyphae. No foot cells were seen in this region.

- 2 - A region shown to produce sporangiophores of stage I development

- 3 - A region shown to have sporangiophores of stage IV

magnification X320

3. Development of sporangiophore from globular branched like structure

magnification X400

FC: Foot Cell

HT: Hyphal Tip

M: Mycelia

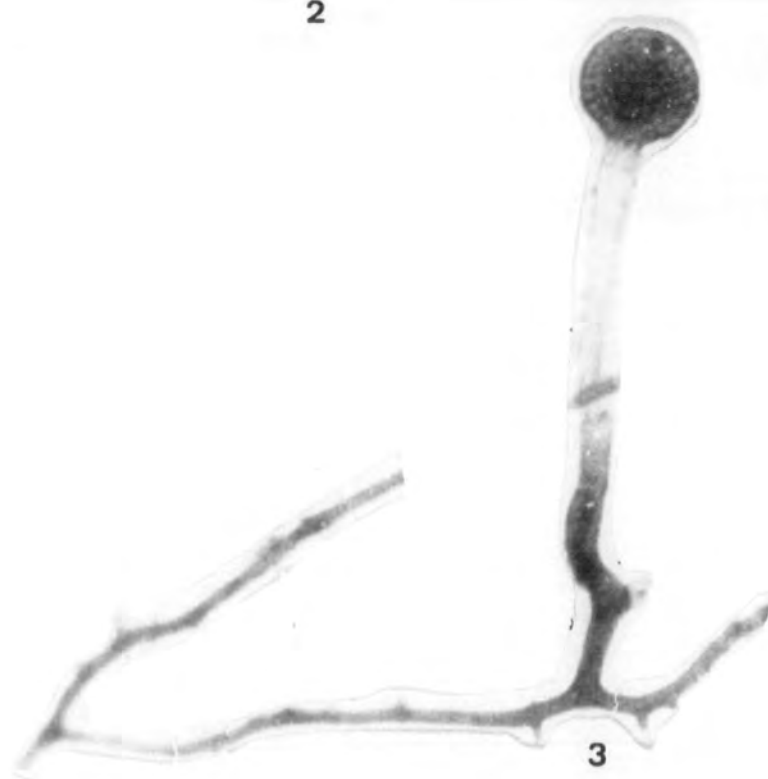
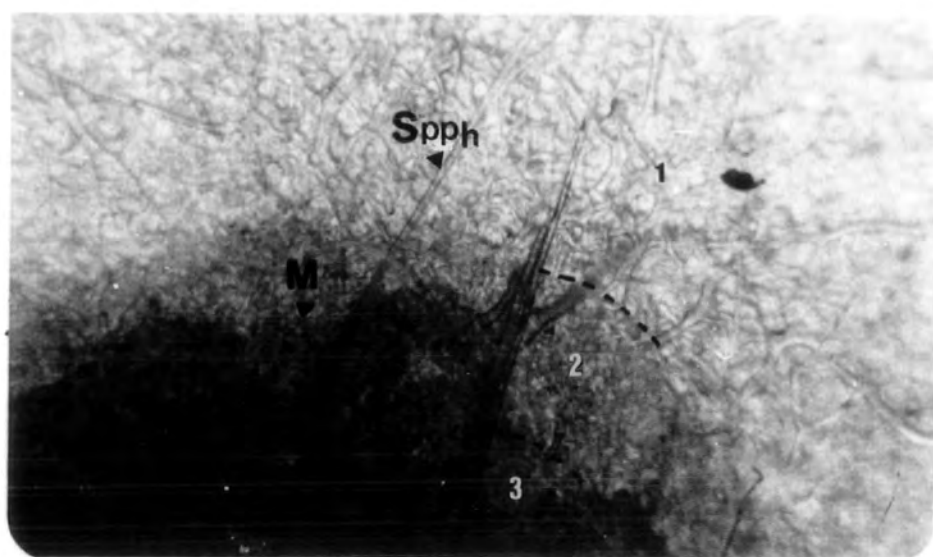
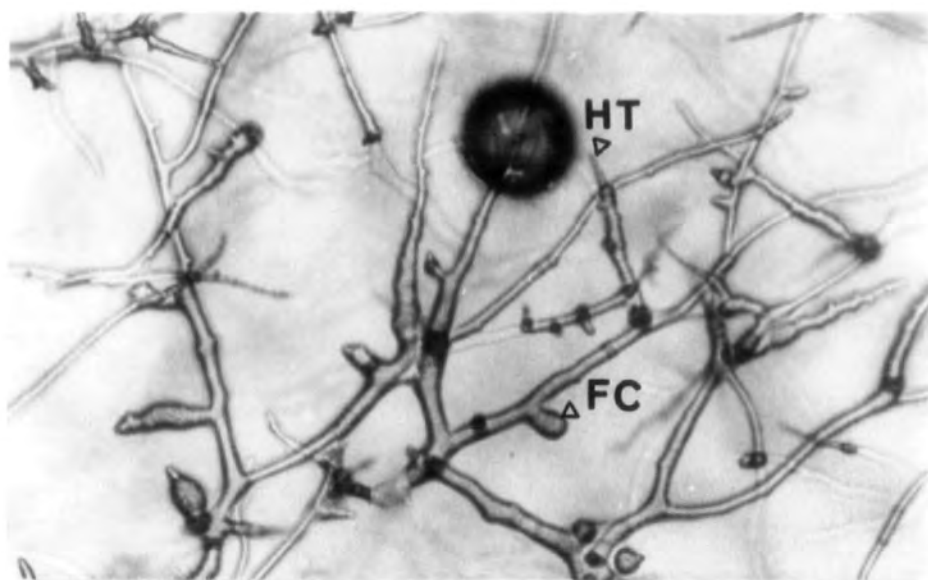


Plate (4)

Different developmental stages of sporangiophore

Stage I

The tapered tip shows greater intensity of

1. RNA
2. Wall binding stain

Sporangiophores were fixed in acetic acid-alcohol and stained with Toluidine blue as shown in (1) and stained with Uvitex BOPT as shown in (2)

- 1 - magnification X1280  
2 - magnification X2500

3-4 Stage II

A - Apex region

Showing swelled tapered tip. Both represent the same stage of development, but shows different colour of sporangiophore and sporangium. Sporangiophores were not stained.

magnification X400

5 Stage III

Showing increase in the size of the sporangium

magnification X400

6-7 Stage IV

Showing morphologically, the change in colour of sporangium compared with stage III. Matured sporangiophore stage IV shows black colour in sporangium.

magnification X320

8-9

Showing wall binding Uvitex BOPT stain for swelling of the tapered region

magnification X1000

9

Overhead view of sporangium, which shows greater wall binding Uvitex BOPT stain

magnification X2850

Cb: Cell wall binding stain  
N: Nuclei  
Sp: Sporangium



1



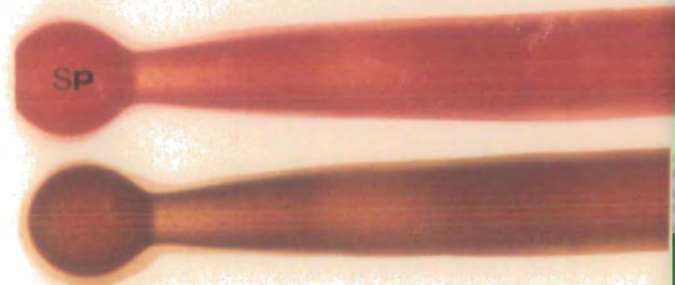
2



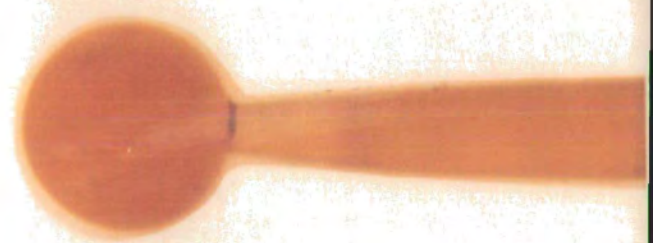
3



4



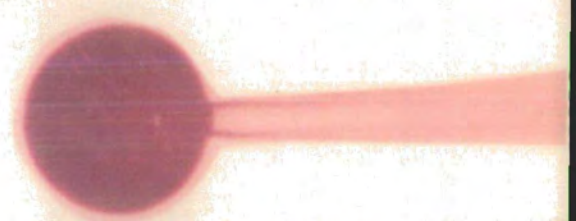
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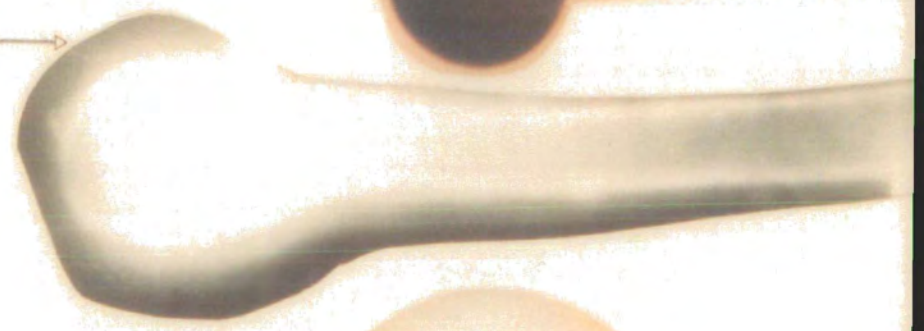
6



7



8



9

Cb  $\Delta$

sp

N  $\Delta$

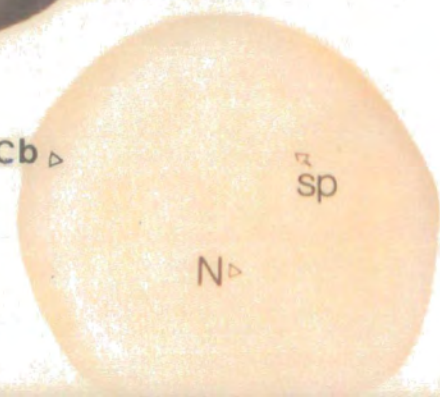


Plate (5)

Distribution of cell wall substances and DNA along the sporangiophore

1. Sporangiophore stained with Uvitex BOPT

magnification X1000

2. Sporangiophore fixed in acetic acid alcohol and stained with Feulgen

magnification X1000

Dotted lines indicate the site of cutting

TIP

BASE

DNA

1

2

Plate (6)

Distribution of nuclei and wall binding stain during the growth and development of sporangiophore. Sporangioophores were fixed in acetic acid-alcohol fixative

1. Stage II
- 2,3. Stage III
4. Stage IV

magnification X3100

C: Columella

cb: Cell wall binding Uvitex BOPT

Ic: Intercalary region

MS: Megaspores

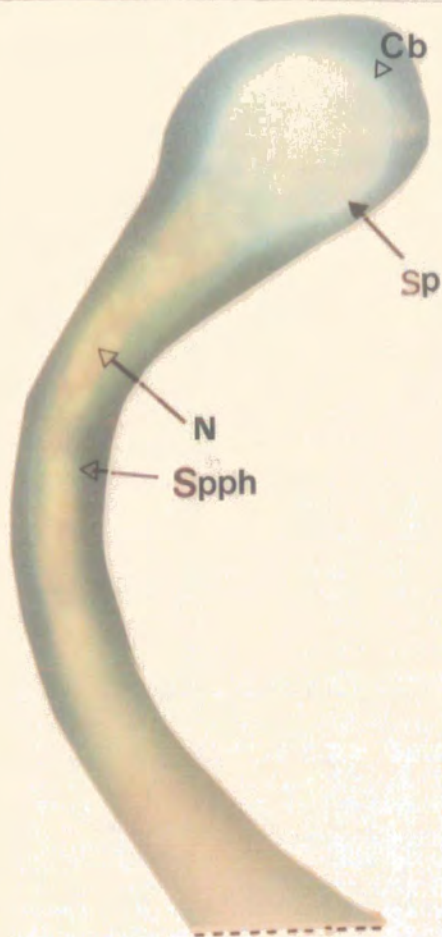
N: Nuclei

Sp: Sporangium

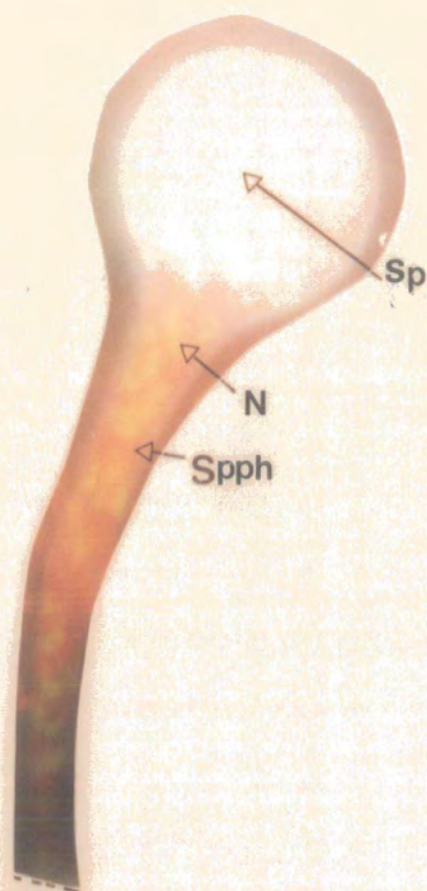
Spph: Sporangioophore

Sporangioophores were stained first by acridine orange and then by Uvitex BOPT.

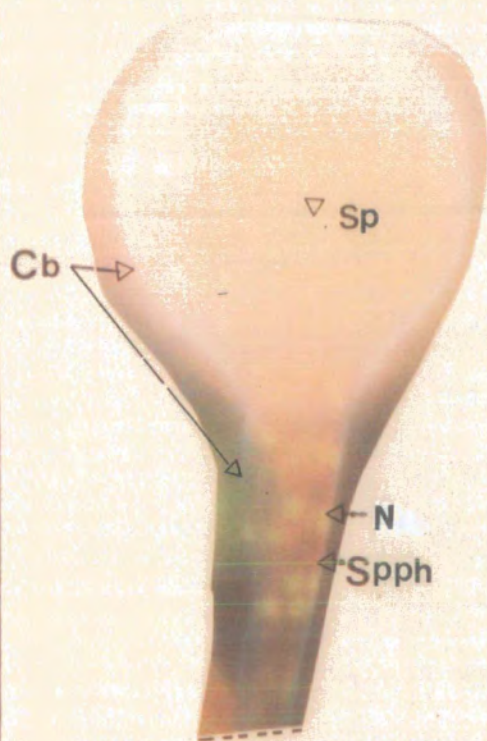




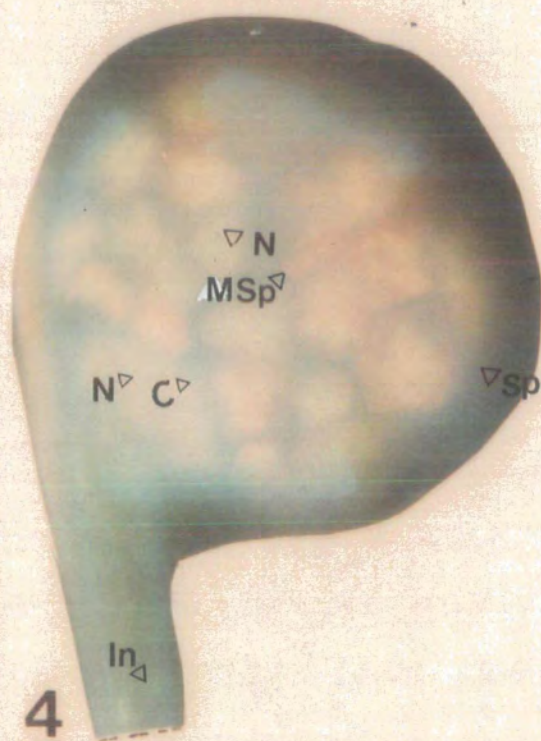
1



2



3



4

Plate (7)

**Distribution of mitochondria along the sporangiophores stained with Blue-Tetrazolium (BT)**

1. Tip region of sporangiophore. The black background indicates the location of mitochondria

magnification X1000

2. Tip region of the sporangiophore stained with BT stain and differentiated with methyl green, 0.15% solution for one minutes. Greater accumulation of mitochondria can be seen at the apex region compared with the DNA

magnification X400

3. Tip region of the sporangiophore stained with BT and showing a greater intensity of stain at the apex region of the sporangiophore

magnification X1280

4. Tip region of the isolated sporangiophore after resuming growth

magnification X1200

A: Apex

C: Cytoplasm

MS: Mitochondria stained intensely

T: Tip region

RW: Regenerated wall which formed after resuming growth of the isolated sporangiophore.  
Normal apex is maintained



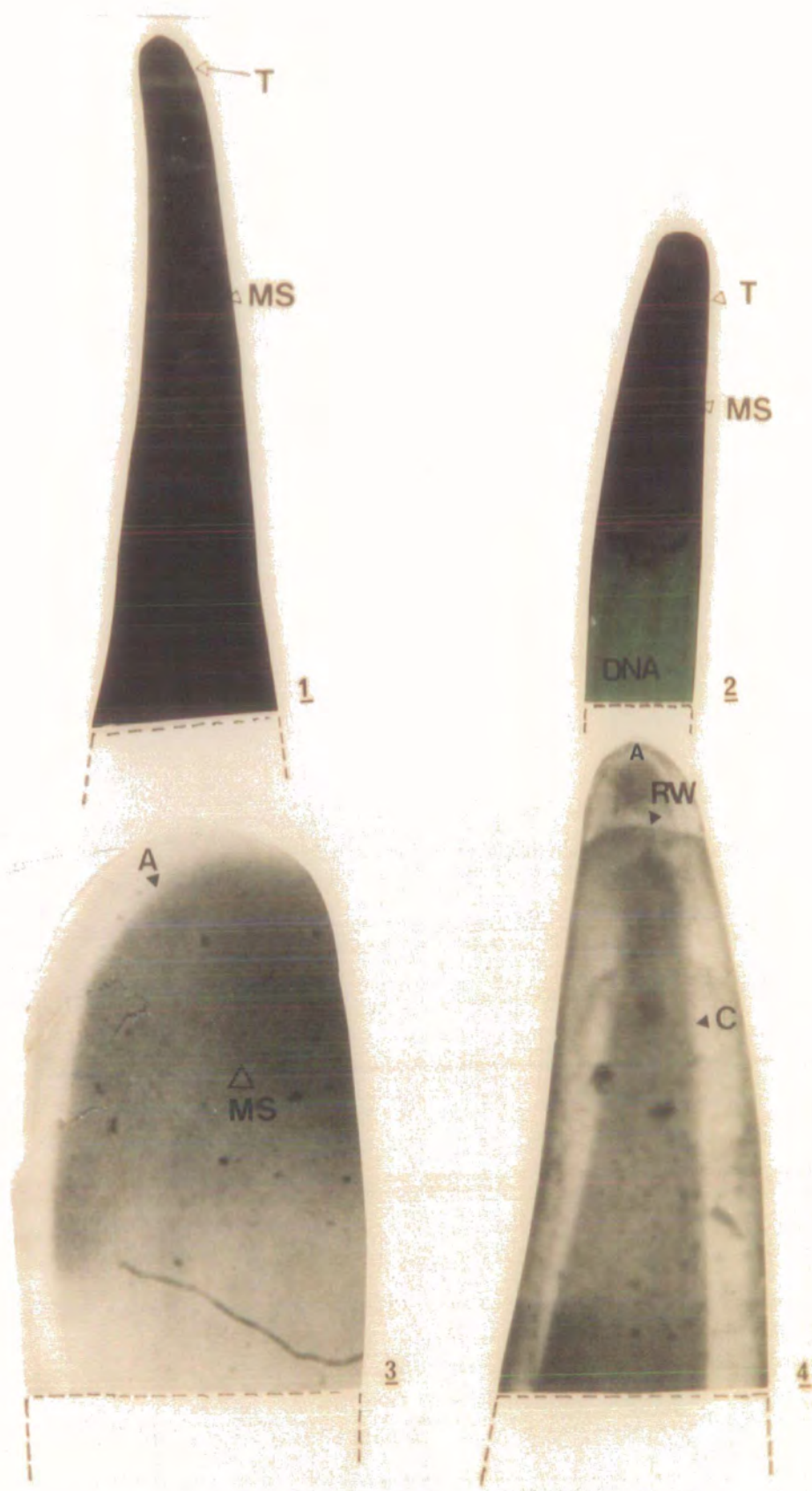


Plate (8)

Distribution of nuclei along stage I. Sporangiphore was fixed with acetic acid-alcohol and stained with Giemsa stain

1. Sub-tip region
2. Middle region
3. Base region

magnification X3200

D: Damage may indicate  
degradation

N: Nuclei

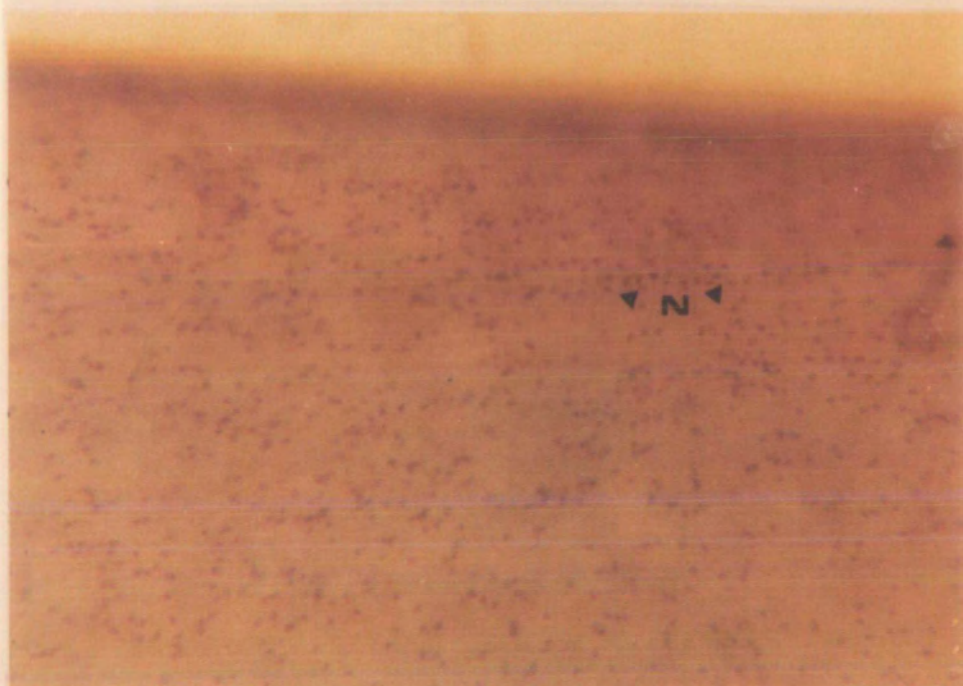
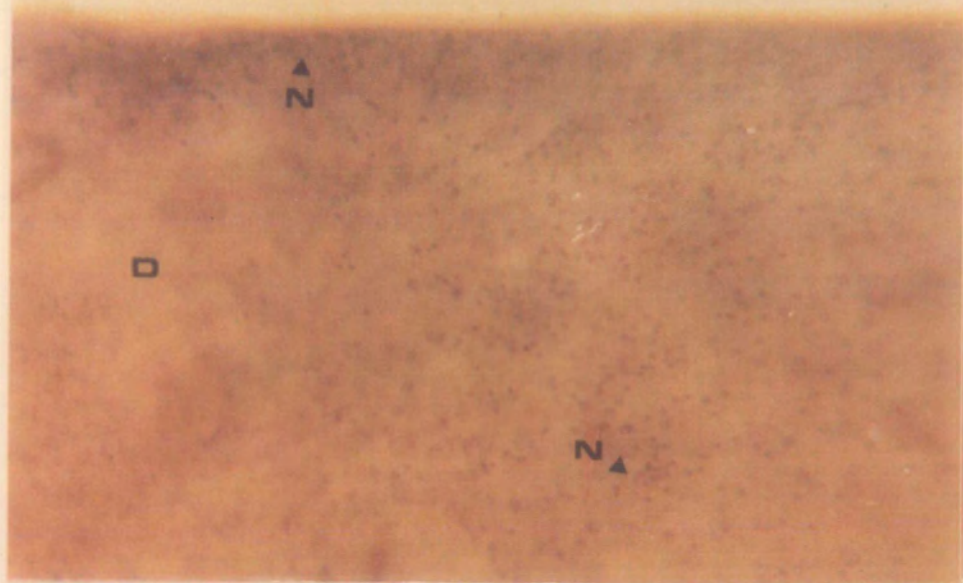




Plate (9)

Distribution of macromolecules along stage I.  
Sporangiophores were fixed in acetic acid-alcohol and stained with Korson's stain

- 1a. RNA distribution along the tip region, pre-treated with  $0.2 \text{ mg.ml}^{-1}$  DNAase before staining  
magnification X400
- 1b. Sporangiphore, pre-treated with  $0.2 \text{ mg.ml}^{-1}$  DNAase and with 0.1% RNAase  
magnification X320
- 2a,b,c. Distribution of RNA and DNA along the sporangiphore  
magnification X320
- 3a,b. RNA distribution along the sporangiphore, pre-treated with  $0.2 \text{ mg.ml}^{-1}$  DNAase  
magnification X400
5. Sporangiphore stained with orange G and showing the distribution of protein  
magnification X320
6. DNA distribution along the sporangiphore, pre-treated with 0.1% RNAase  
magnification X320
- 7a,b,c DNA distribution along the sporangiphore, pre-treated with 0.1% RNAase  
magnification X320

See page 42 for details

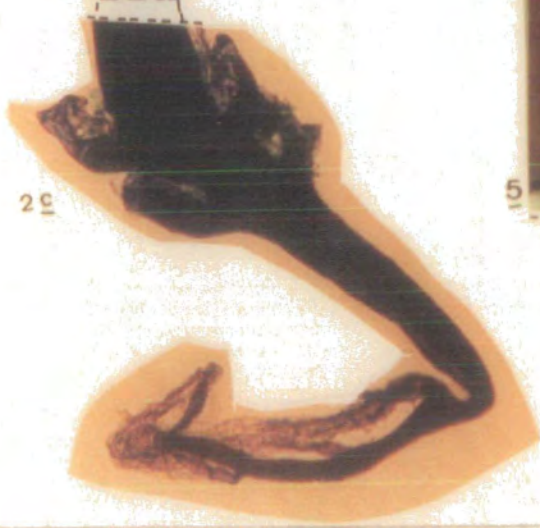
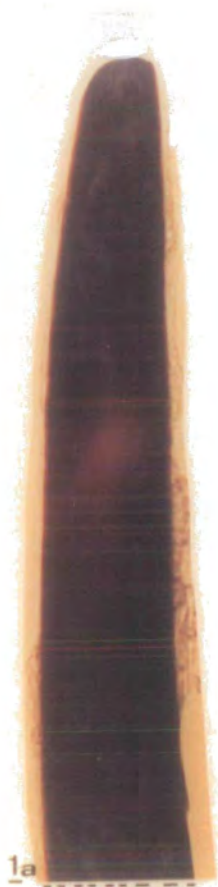


Plate (10)

Distribution of nuclei along the sporangiophore, fixed in acetic acid-alcohol and stained with Feulgen stain

magnification X320

OB: Obconical Base

Na: Nuclei accumulation at the apical region

Pe: Peripheral region, surrounding the vacuole

V: Vacuole



A  $\Delta$  Na

$\Delta$  Pe

V

DNA

OB  
 $\Delta$

3

Plate (11)

Distribution of nuclei at the tip region with high magnification. Sporangiphore was fixed in acetic acid-alcohol and stained with Feulgen stain

magnification X7000

N: Nuclei

V: Vacant zone or Exclusion zone



Plate (12)

Morphological changes during regeneration of segments on the solid medium

1. Tip, middle and basal regions after regeneration and development of sporangiophores

magnification X50

2. Regeneration of isolated sporangiophore with cut apical region. Sporangiophore was cultured in liquid minimal medium

magnification X33

3. Regeneration of isolated sporangiophore, cultured on solid medium. This shows the regeneration and development of sporangiophores from both regions

magnification X33

4. Regeneration of isolated sporangiophore, cultured in water

magnification X26

a: apical site with cut apex region

b: basal site

s: sporangium

M: Mycelium



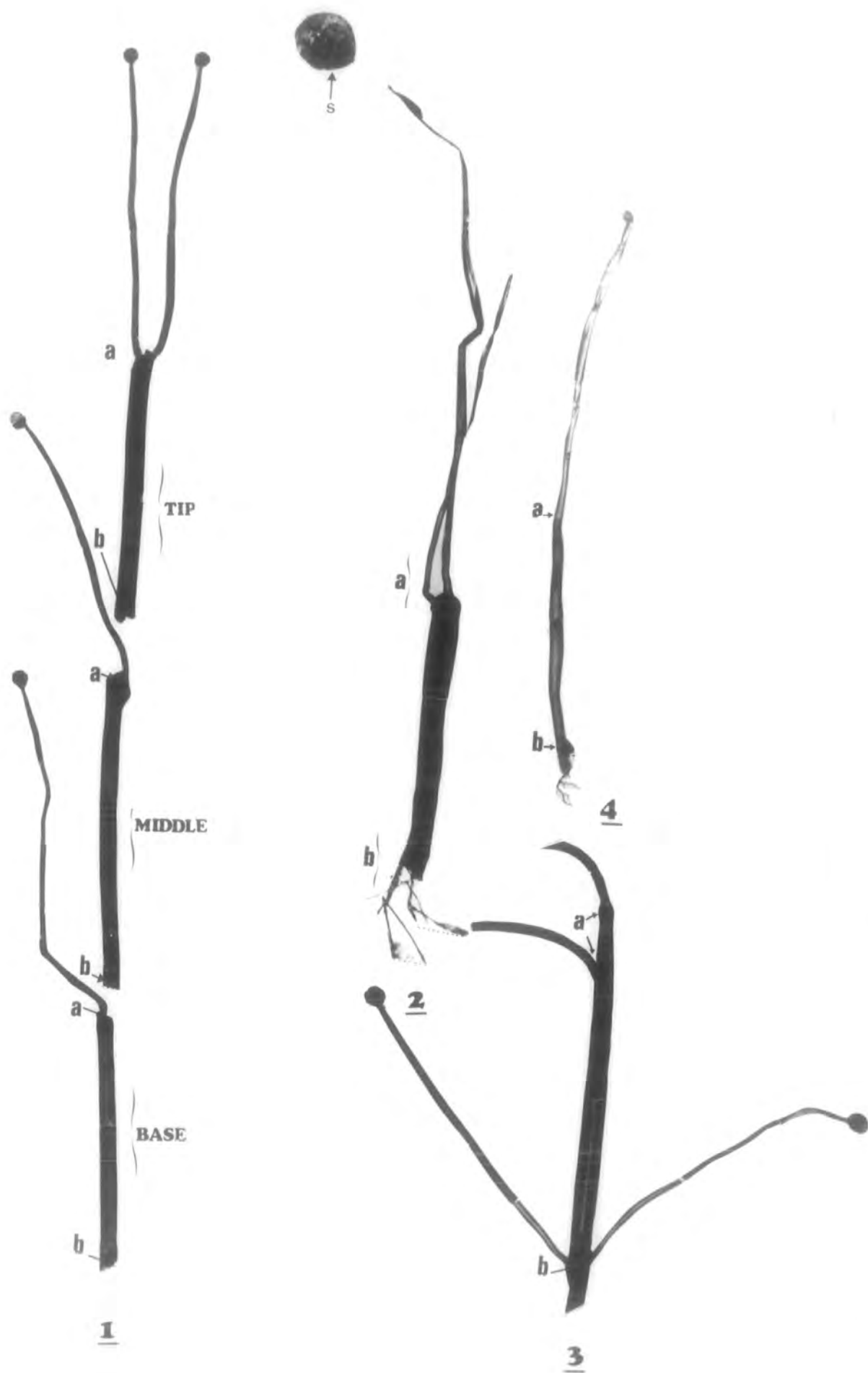


Plate (13)

Regeneration and development of isolated sporangiophores  
cultured on the solid minimal medium

1. Development of two sporangiophores from the basal  
end

magnification X100

2. Development of one sporangiophore from the basal  
end

magnification X100

3. Development of sporangiophores from both cut ends

magnification X100

4. Development of sporangiophores from the apical  
region, and exhibiting strong phototropic response

magnification X100

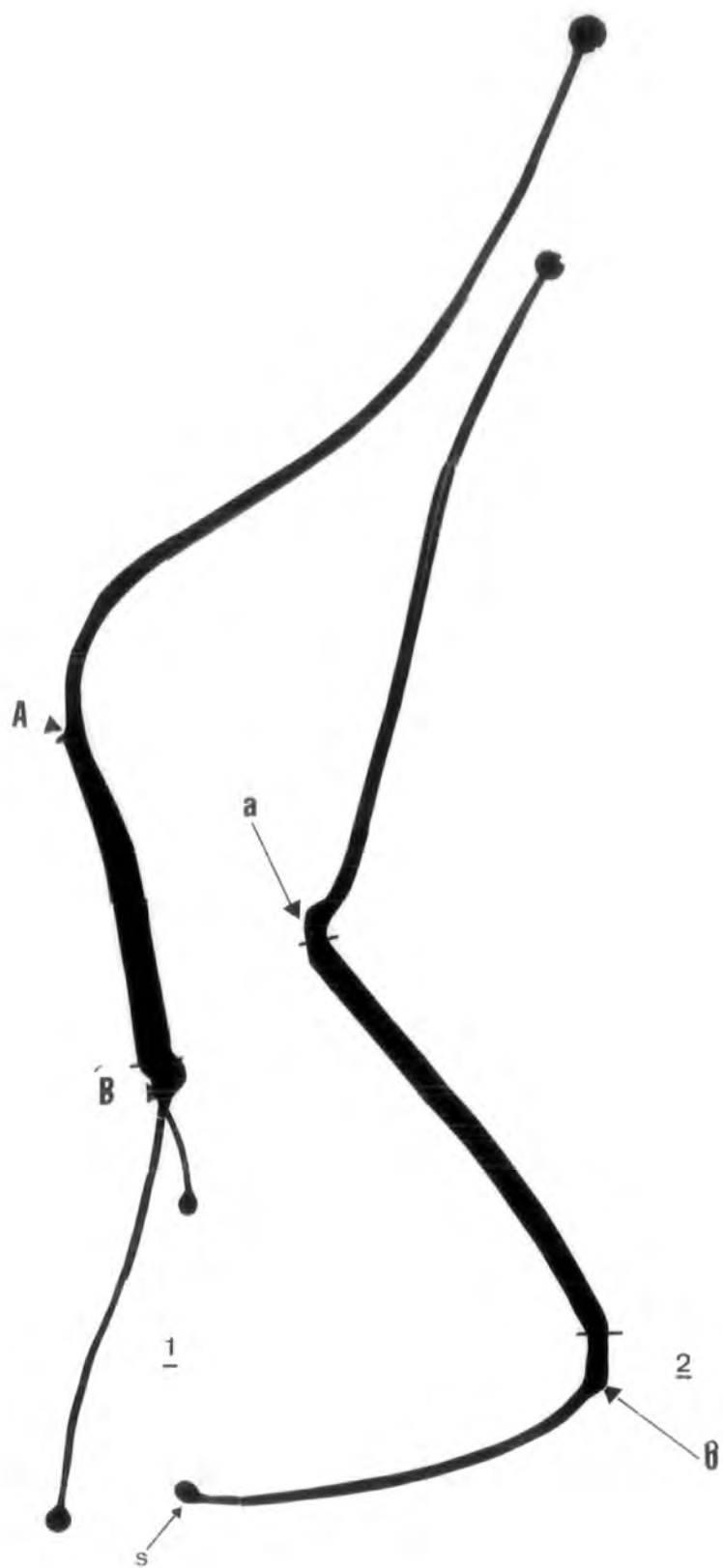
A: Apical region

a: apical region after resuming  
growth

B: Basal region with two regenerated spph.

B: basal region with one regenerated spph.

S: Sporangium



TIP  
SITE

Spph

Spph

Spph

BASAL  
SITE

3

4

Plate (14)

Regeneration and development of sporangiophores which were cultured on the solid minimal medium

1. Regeneration and development of sporangiophores from the tip with cut apex region  
magnification X52
2. Regeneration and development of sporangiophores from the basal region  
magnification X100
3. Regeneration and re-regeneration of the sporangiophore from the tip region  
magnification X100
4. Regeneration and development of mycelia and sporangiophore from the basal and tip regions respectively. The regenerate sporangiophores from the tip region exhibiting phototropic response to overhead illumination  
magnification X100
5. Regeneration and development of sporangiophores from both ends of tip and basal regions. The sporangiophore which emerged from the tip and exhibiting phototropic response to overhead illumination  
magnification X100
6. Regeneration and development of sporangiophore at the upper part of the stump on the attached sporangiophore, i.e. below the ligation  
magnification X100

L: Ligation

Re: Regenerated sporangiophore

Rr: Re-regenerate sporangiophore





1



2



3



4



5



6

Plate (15)

Distribution of nuclei during resuming growth at the extreme apex of the isolated sporangiophore. Sporangiophores were fixed in acetic acid - alcohol and stained by Feulgen stain.

1. Nuclei distribution at 0h after isolation

magnification X1100

2. Nuclei distribution at 8 hrs of culturing of the sporangiophore in No. (1)

magnification X1100

A: Apex region

GrR: Growing region after  
resuming growth

MN: Accumulation of nuclei



1



2

Plate (16)

Distribution of wall binding stain (Uvitex BOPT) during  
regeneration and development

1. Isolated sporangiophore was cultured in liquid  
minimal medium. It shows more intensity of stain  
at the apex and basal regions of the sporangiophore

magnification X2800

A: Apex

B: Base

2. During the development of hyphae from the basal  
region

magnification X5000

3. High magnification of the basal region of the  
sporangiophore (No. 1)

magnification X5000

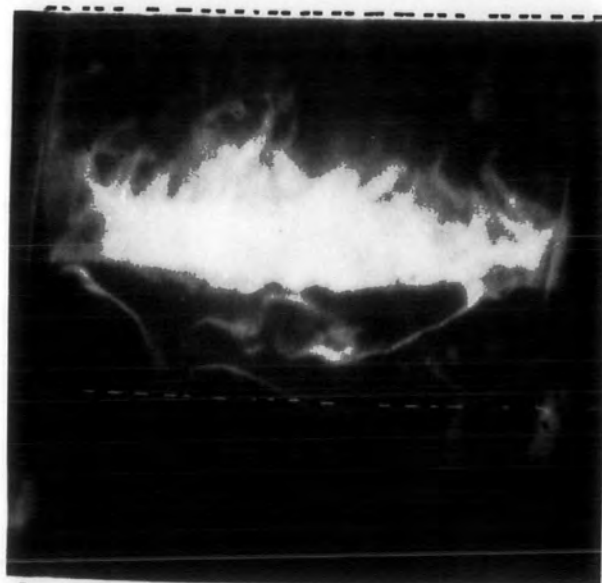
D: Differentiated Hyphae

HT: Hyphal main Tip

Re: Regeneration



2



3

1



Plate (17)

Branch formation after isolation of sporangiophores.  
Sporangiophores were fixed in acetic acid alcohol.

1. Presence of branches emerging from the region below the apex. Growth also resumed by the extreme apex. Sporangiophores were stained by Giemsa

magnification X1280

- 2,3 Branch formed at the sub-apical region with completed apex (1) and with cut apex regions (2). Sporangiophores were pre-treated with DNAase, fixed with acetic acid-alcohol and stained with Korson's stain. The basal end of the branched sporangiophore shows a greater intensity of stain for RNA

magnification X1280

4. The apical tip of the sporangiophore with branches at the sub-apical region; no accumulation of cellular components after staining with orange G. Greater intensity of the stain can be seen in the sporangiophore branches

magnification X1280

A: Apex

Ac: Accumulation of stain or bud  
scar

Br: Branch

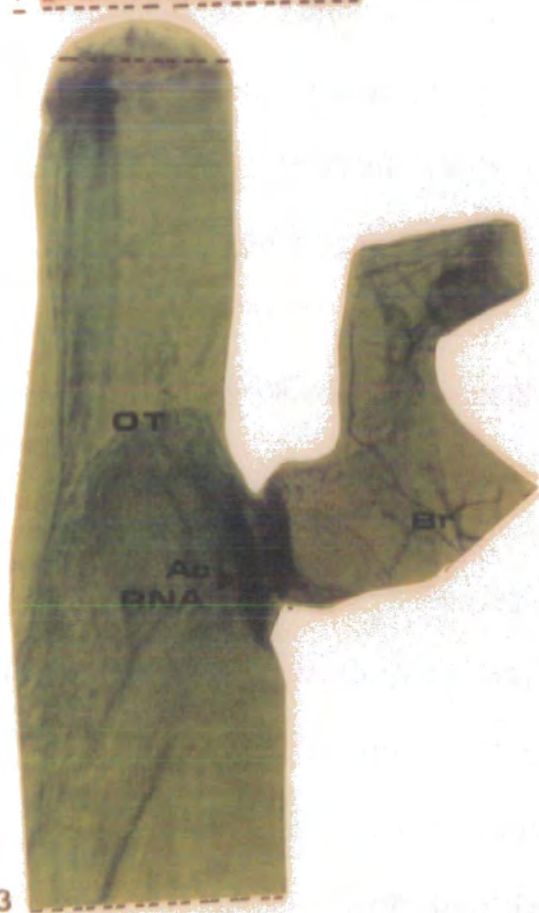
OT: Original Tip



1



2



3



4

Plate (18)

Distribution of cell wall binding stain during growth of the isolated sporangiophore

1. Sporangiophore growth zone, stained with Uvitex BOPT

magnification X2187

GZ: Growth zone

2. During regeneration of the sporangiophore from the sub-apex region. The regenerated sporangiophore stained intensely with the Uvitex BOPT

magnification X1280

OT: Original Tip

RP: Regenerated protoplast

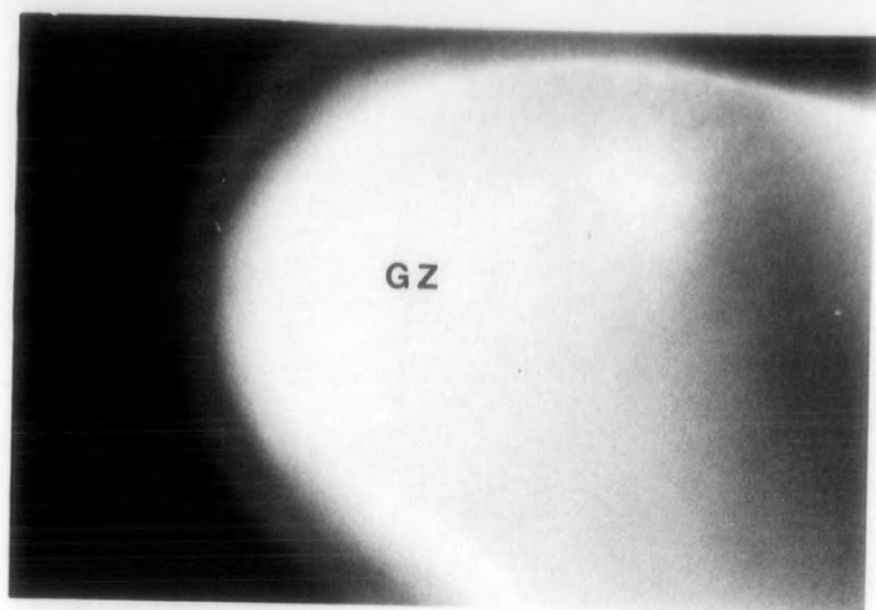
3. During regeneration of hypha through the basal end of the sporangiophore, cultured in liquid minimal medium

magnification X2187

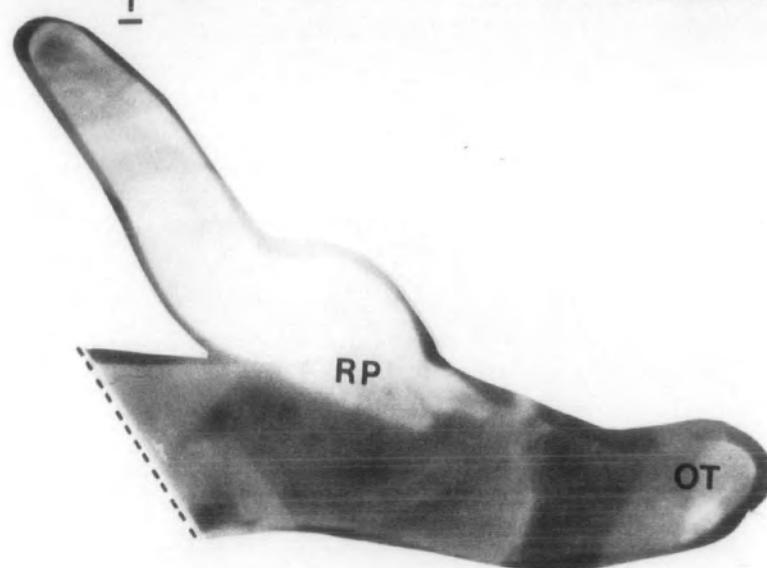
RH: Regenerated Hypha

SB: Sporangiophore Base

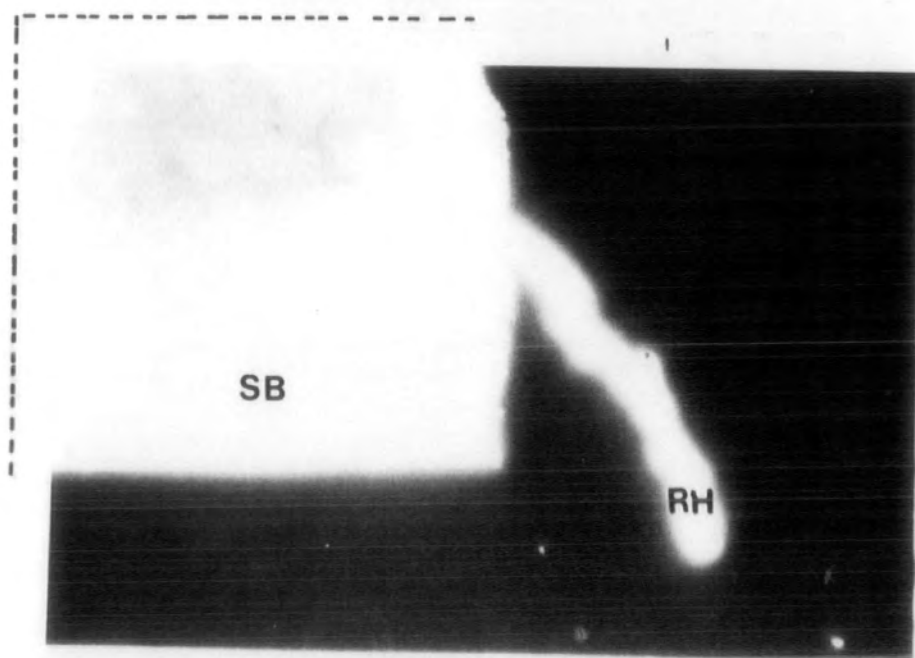




1



2



3

Plate (19)

Distribution of protein and nuclei after regeneration of the isolated sporangiophore, with cut apex regions. Sporangiophores were fixed in acetic acid-alcohol

1. Distribution of protein after 4 hours
2. Distribution of protein after 10 hours
3. Distribution of protein for separate sporangiophores at 1-2 hours. Sporangiophores in 1,2,3 were stained with Orange G.

Distribution of nuclei after 2 hours. Giemsa stain was used

magnification X1280

- 4,5. Distribution of nuclei after 3 hours. Giemsa stain was used

magnification X3200

N: Nuclei

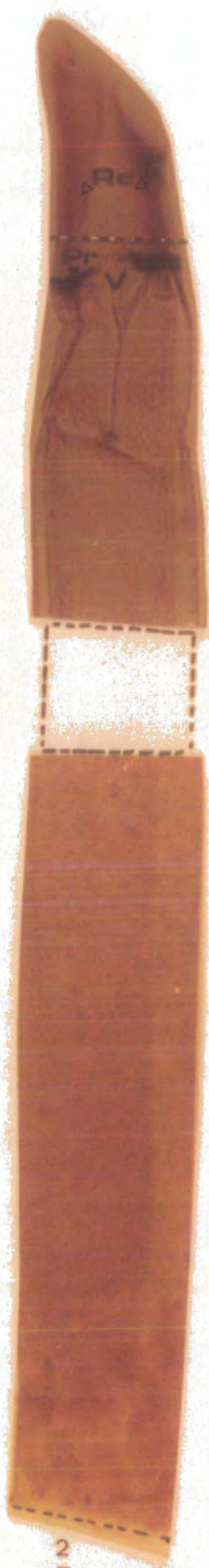
Pr: Protein accumulation

Re: Regeneration

V: Vacuole



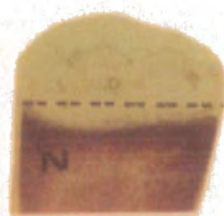
1



2



3



4



5

Plate (20)

Distribution of wall binding stain (Uvitex BOPT) during regeneration of sporangiophore with cut apex regions (1,3,6).

1. Showing formation of several branches

magnification X1250

2. Showing swelling region after regeneration

magnification X1250

3. Resuming of apical growth

magnification X2500

6. Branch formation

magnification X2500

4. Distribution of protein after ligation,  
Sporangiophore was fixed in acetic acid-alcohol  
and stained with orange G

magnification X500

5,7. Distribution of DNA and RNA after ligation of the  
tip region. Sporangiohores were fixed in acetic  
acid-alcohol and stained with Korson

5. Isolated sporangiophore

6. Attached sporangiophore

magnification X500

Br: Branch

L: Ligature place

Pr: Protein

RT: Regenerated Tip

Sw: Swelling area

Re: Regeneration



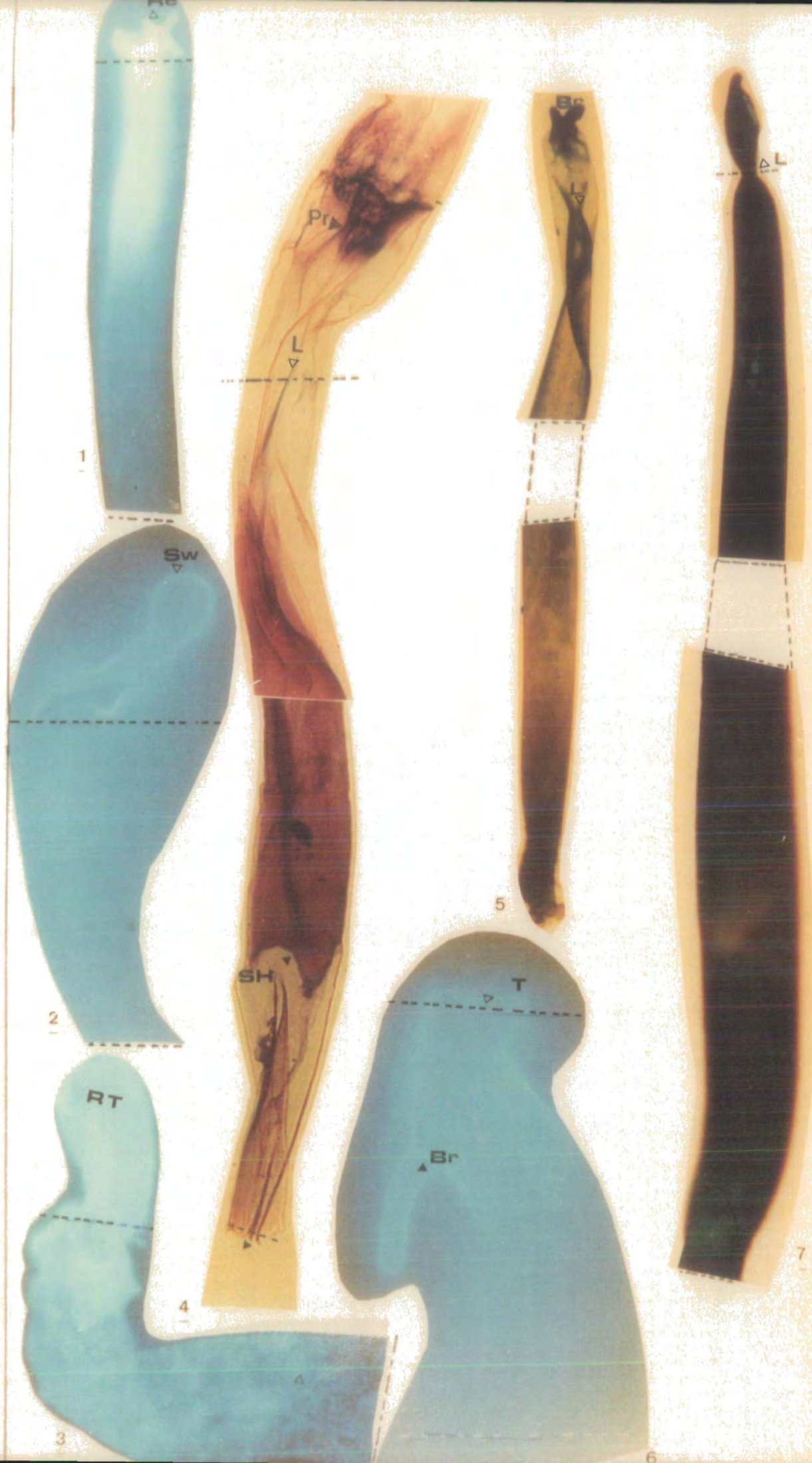


Plate (21)

1 & 2. Distribution of RNA, DNA and Protein of stump

1. 2 hr after excising apex region

2. 8 hr after excising and regeneration

magnification X1280

Sporangiophores were fixed in acetic acid-alcohol and stained with Korson

3. Distribution of RNA, DNA and Protein 8 hr after excising apex region

magnification X500

Fixation and staining as in (1 & 2)

4. Distribution of Nuclei along the isolated sporangiophore 8 hr after excising apex. Sporangiophore was fixed in acetic acid-alcohol and stained with Giemsa

magnification X820

Auto: Autolysis or Degradation

N: Nuclei

SH: Shifted or elevated base

V: Vesicles

WH: Wound Healing

BASE

TIP

AUT.

DNA  
+  
PROT.

DNA

RNA

P<sub>1</sub>

SH

V

N

WH

2

3

4



Plate (22)

Distribution of nuclei in the basal region of the sporangiophores. Sporangiophores were fixed in acetic acid-alcohol and stained with Giemsa stain

1. Stump on attached sporangiophore

magnification X2560

2. Comparable region of isolated sporangiophore

magnification X2650

D: Damage or degradation

N: Nuclei



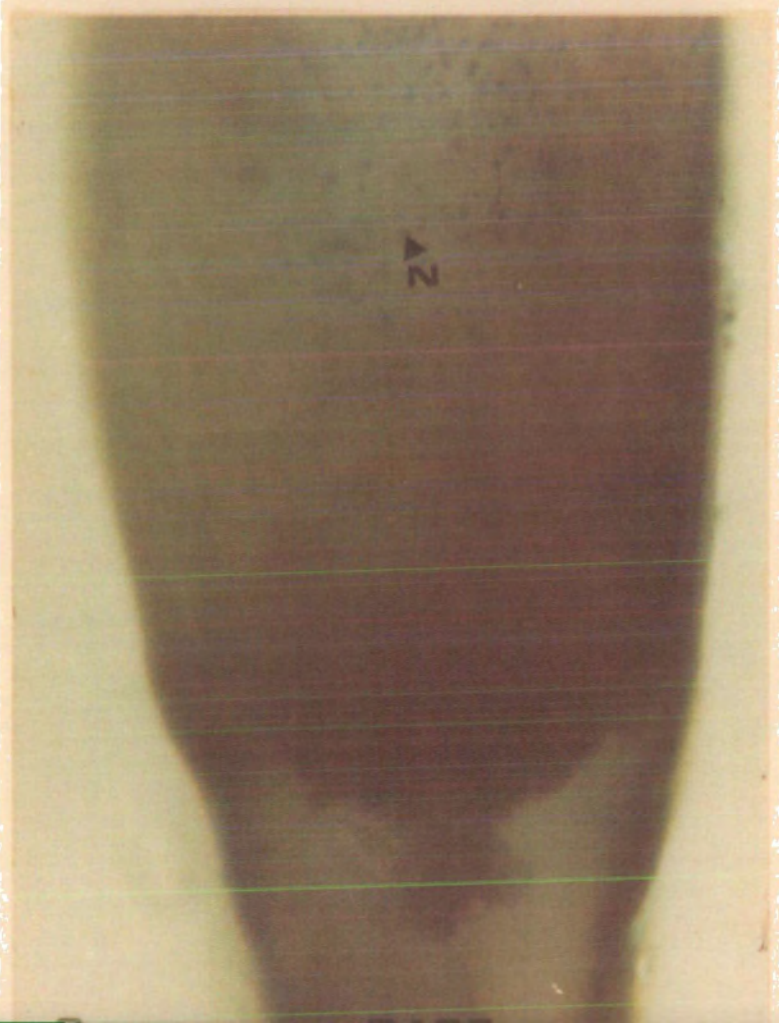


Plate (23)

Distribution of nuclei at the basal regions.  
Sporangiophores were fixed in acetic acid-alcohol and  
stained with Giemsa

- 1 & 2. Showing nuclei distribution of the basal regions  
of stump on attached sporangiophores

magnification X4000

3. Showing nuclei distribution at the basal region  
of comparable isolated sporangiophore

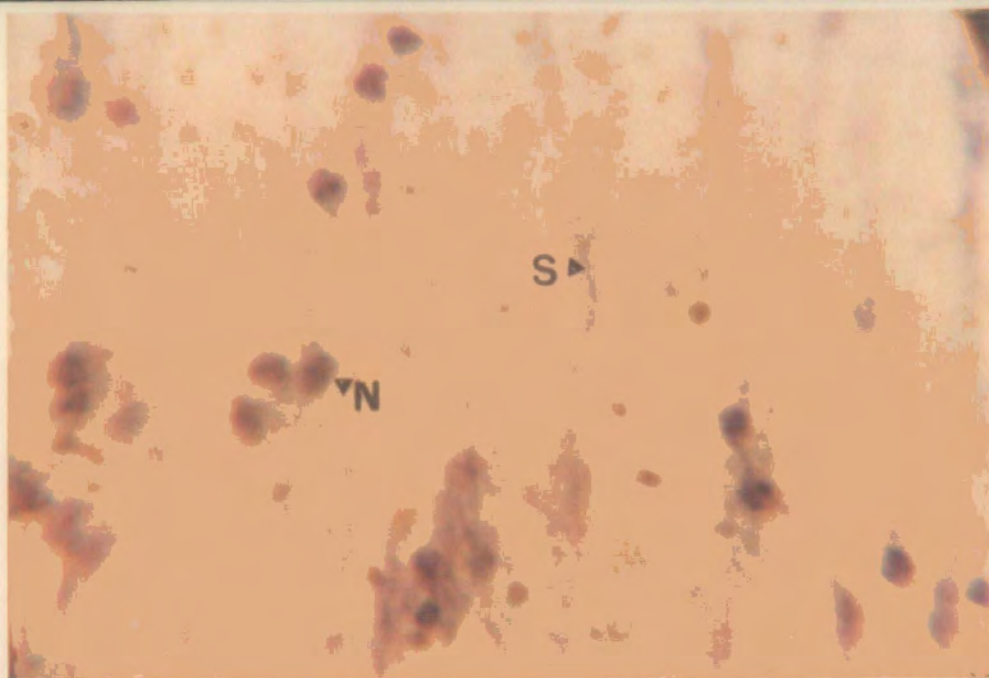
magnification X4000

The direction of movement of nuclei, shown  
with straight strands along the axis of the  
sporangiophore

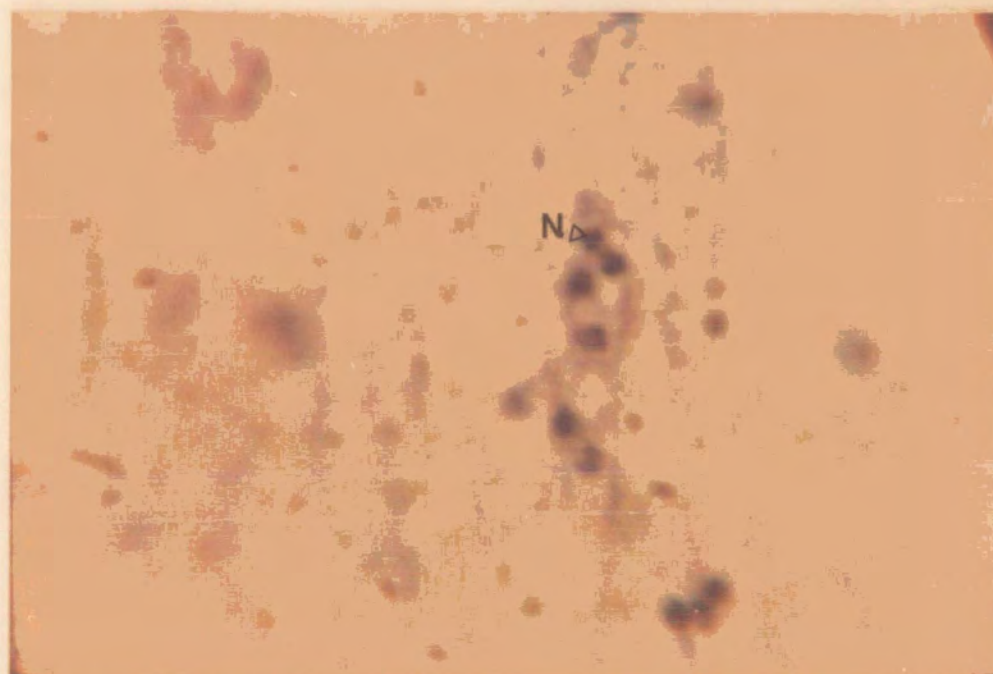
N: Nuclei

S: Strand

1



2



3

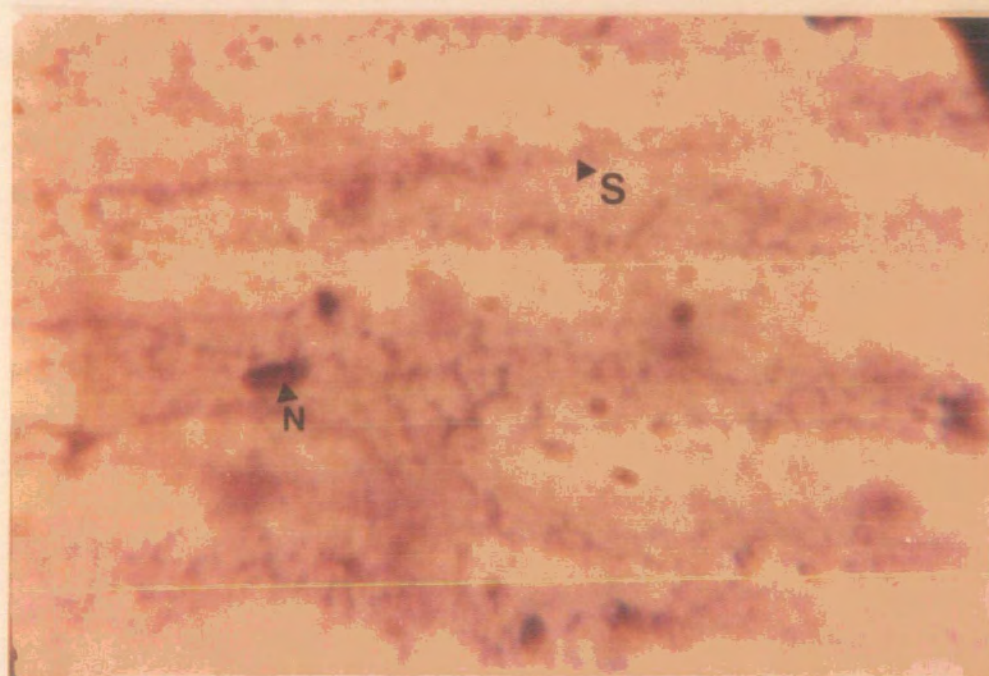


Plate (24)

Distribution of nuclei, vesicles and RNA during regeneration of the isolated sporangiophore. The sporangiophore was cultured without apex region, and was fixed in acetic acid-alcohol and stained with acridine orange

1. Distribution of nuclei, vesicles and RNA at regenerated site of apical region

magnification X4000

2. Distribution of nuclei and RNA along the tip region after regeneration

magnification X4000

3. Distribution of nuclei and RNA at the region below region 2

magnification X4000

4. Distribution of nuclei, vesicles and RNA at the basal region after regeneration

magnification X4000

Ea: End of the regenerated apical region

N: Nuclei

Ve: Vesicles like structure



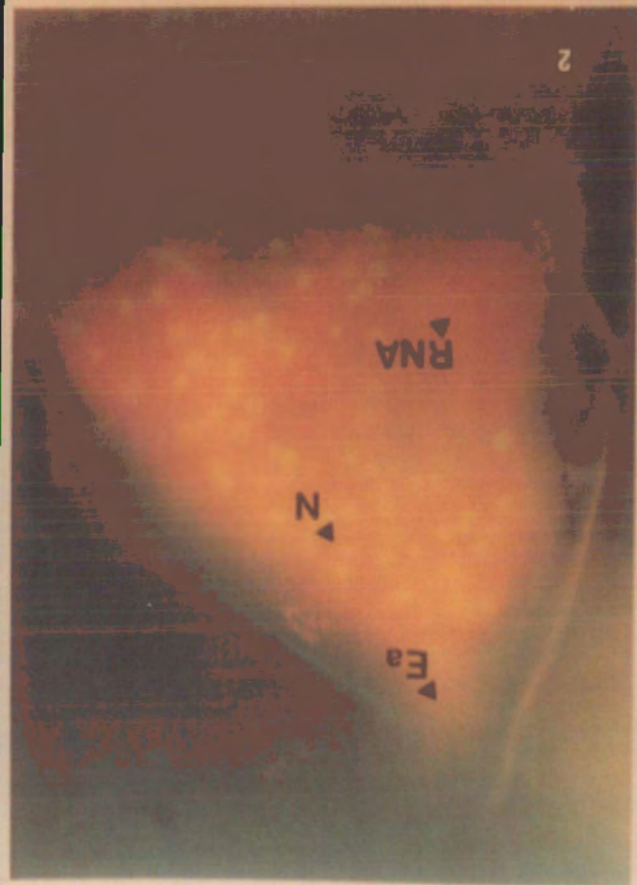
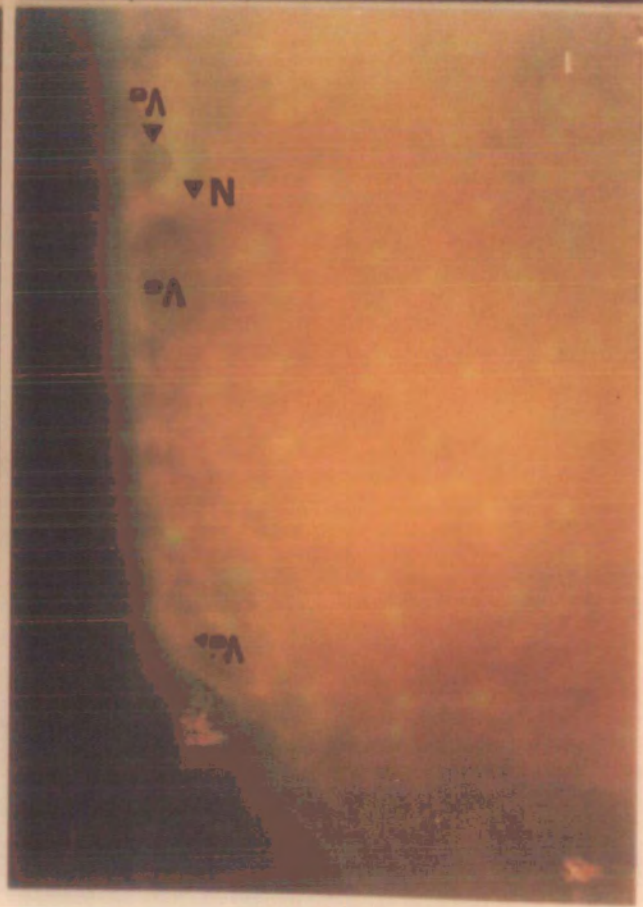
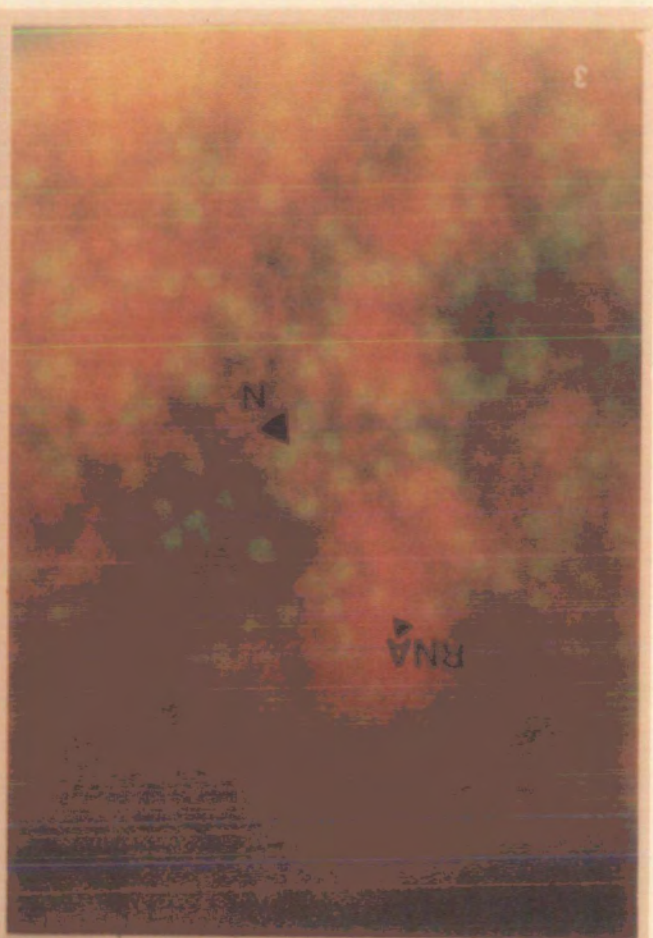


Plate (25)

The effect of the mechanical incision on the distribution of nuclei. Sporangiphores were fixed in acetic acid-alcohol and stained with Giemsa stain

1. Before incision
2. After incision, greater accumulation of nuclei can be seen at the injured area

magnification X3200

CS: Current Strands

In: Incision

N: Nuclei

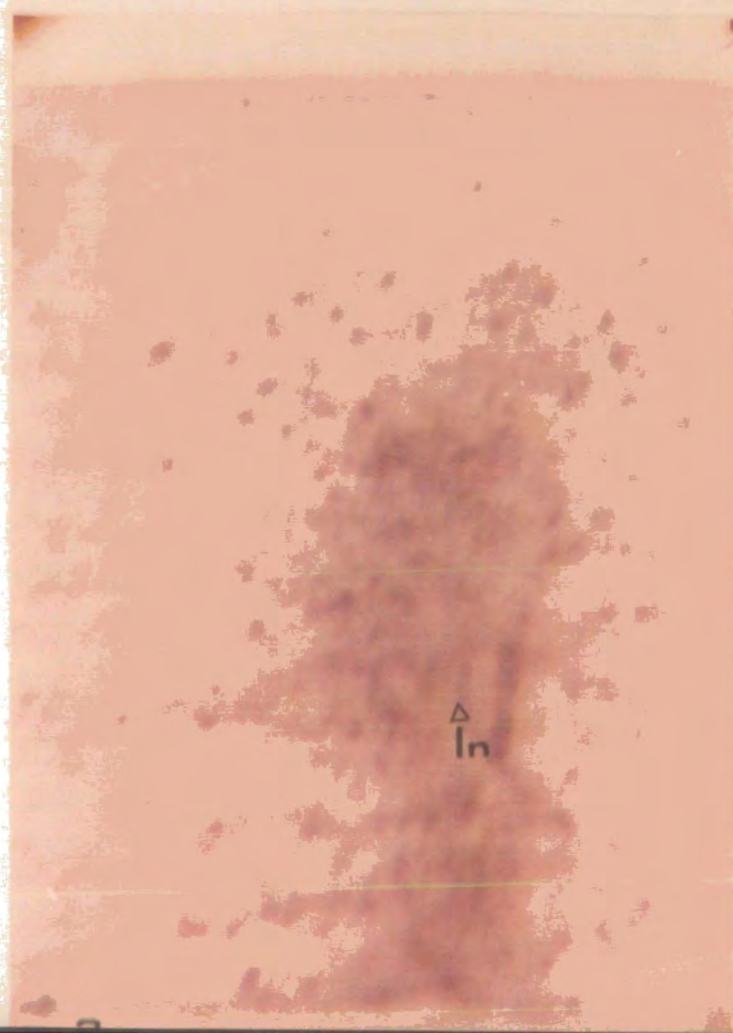
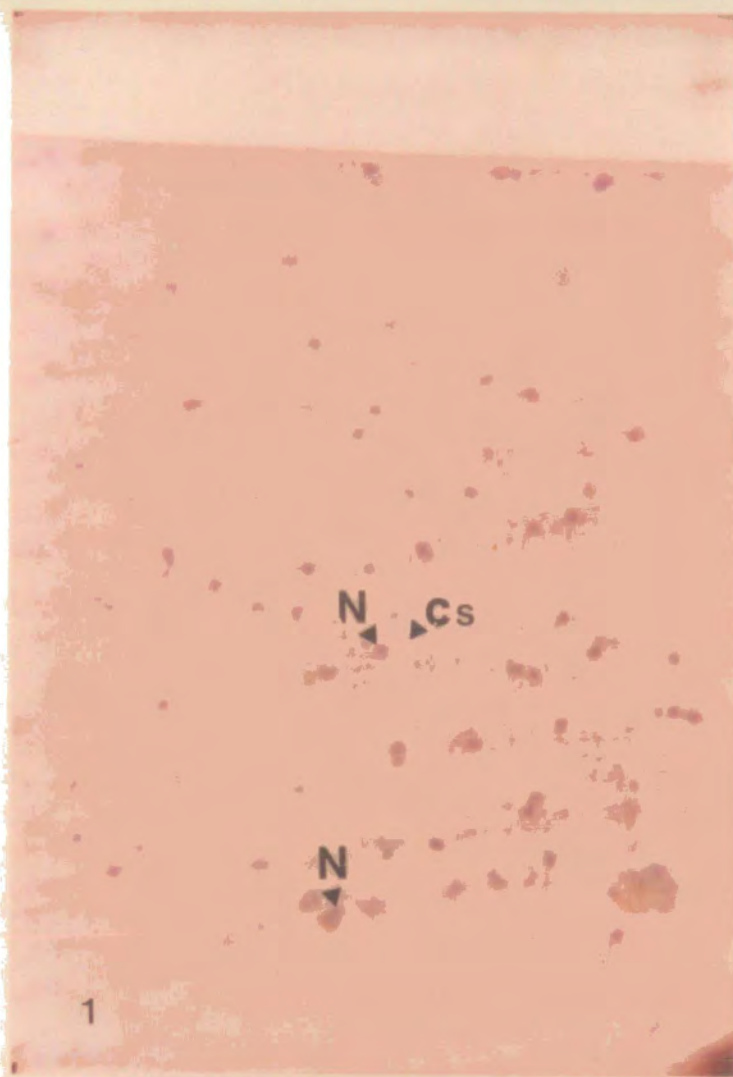


Plate (26)

Morphological changes during regeneration of segments,  
cultured on the solid medium

magnification X320

A - Tip with apical segment

o

A - Sub-apical segment

M - Middle segment

SM - Sub middle segment

B - Basal segment

a - apical site

b - basal site

A' - Tip segment with damaged apical region

A'' - Tip segment with intact apical region, but  
showing branch formation which emerges from  
the sub-apical region

<sup>2</sup>  
A<sub>1</sub> to 4 - Sub-apical segments showing location and  
number of regenerated sporangiophores at the  
upper part of the segments





Plate (27)

Distribution of RNA during regeneration of tip segments. Segments were fixed in acetic acid-alcohol and stained with Korson's stain

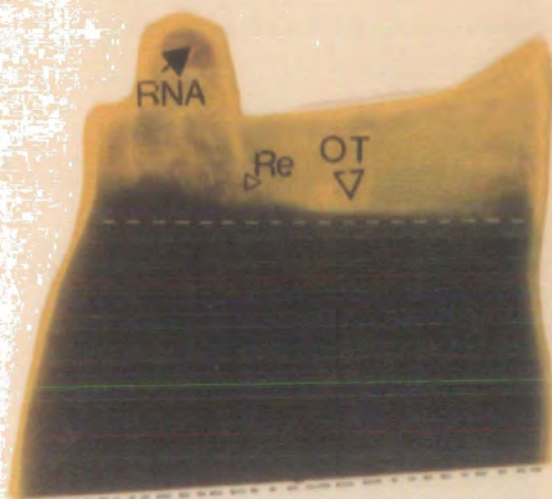
1. After 2 hours of cutting  
  
magnification X1280
2. After 2 hours of cutting and pre-treated with  
0.2 mg.ml<sup>-1</sup> DNAase before staining  
  
magnification X1280
3. After 4 hours of cutting and pre-treated with  
0.2 mg.ml<sup>-1</sup> DNAase before staining  
  
magnification X1280
4. After 10 hours of cutting and pre-treated with  
0.2 mg.ml<sup>-1</sup> before staining  
  
magnification X1280
5. After 8 hours of cutting  
  
magnification X1800

OT: Original cut Tip site

Re: Regeneration

T: Tip region

V: Vesicle



2

3

4

1

5

Plate (28)

Distribution of DNA and nuclei during regeneration of tip segment. Segments were cultured on solid media. They were fixed in acetic acid-alcohol

1. After 2 hours; stained with Korson's stain and pre-treated with 0.1% RNAase before staining

magnification X3100

2. After 4 hours; stained with Feulgen stain

magnification X1280

3. After 4 hours; tip segment with compled apex, stained with Giemsa stain

magnification X2560

4. After 10 hours; stained with Korson's stain and pre-treated with 0.1% RNAase before staining

magnification X6000

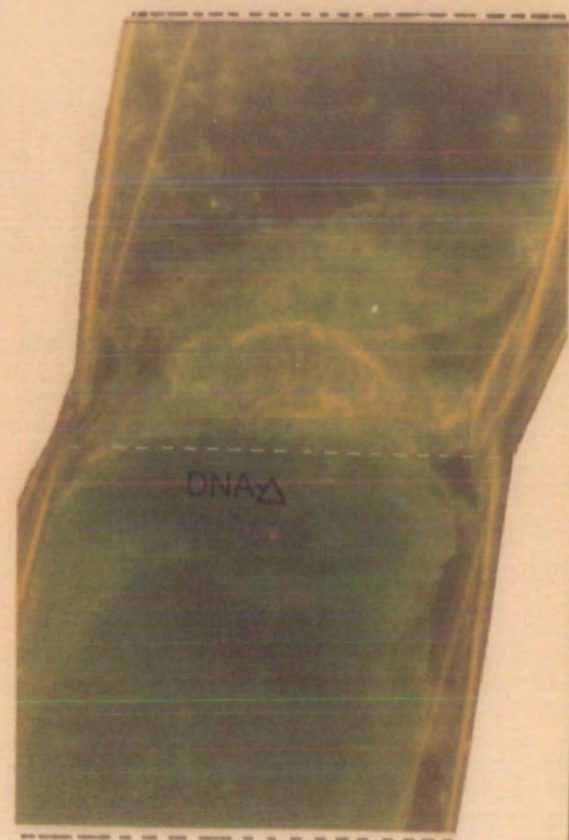
A: Apex region

T: Tip segment

U: Unknown body

V Vacuole





2

3

4

Plate (29)

Distribution of macromolecules after regeneration of segments

Segments were cultured on solid media and fixed in acetic acid-alcohol

- a. RNA distribution along the middle segment after 20 hours regeneration. Segments were stained with Korson's stain

magnification X370

- b,c,c. Distribution of protein 2, 12 and 24 hrs after regeneration of middle segments. Segments were stained with orange G.

magnification X320

- d,e. Distribution of RNA and DNA after 14 and 24 hrs regeneration of basal segments. Segments were stained with Korson's stain

magnification X320

- f,g,h,i. Distribution of protein 14, 16, 18 and 24 hrs after regeneration of basal segments. Segments were stained with Korson's stain

magnification X320

- j. Distribution of protein 4 hrs after regeneration of the tip segment. Segment was stained with orange G

magnification X400

br: branch

deg: degradation

Pr: Protrusion

pro: protein

re: regeneration

Dotted lines indicate site of cut sporangiophores

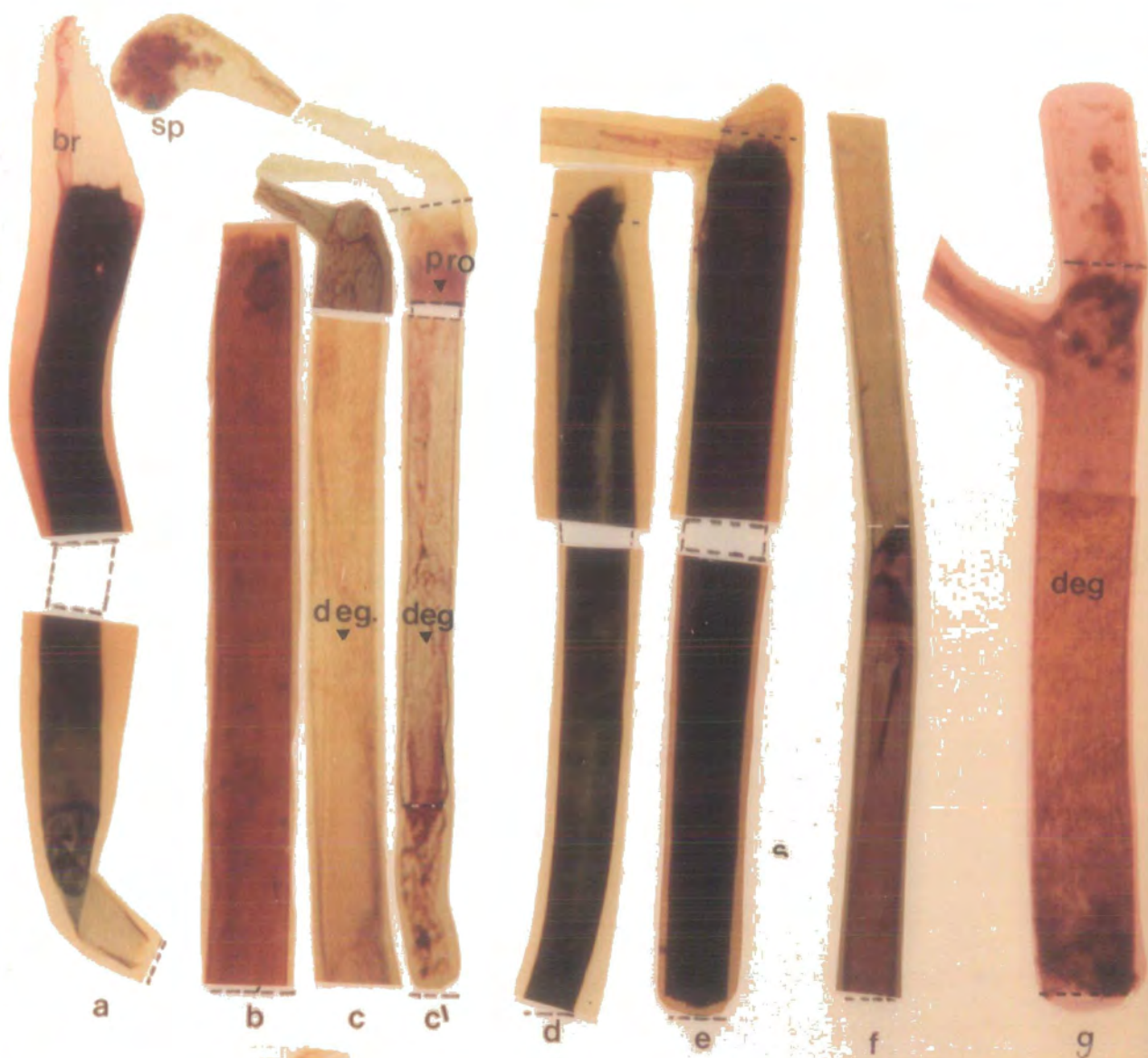




Plate (30)

Distribution of nuclei during regeneration of tip segments. Segments were fixed in acetic acid-alcohol and stained with Giemsa

1. After 4 hrs

magnification X1000

2. After 4 hrs

magnification X3200

3,4. Regeneration and development of sporangiophore at the apical end of tip segment after 12 hrs

magnification X1250

magnification X3200

Ac: Accumulation of stain indicates nuclei

An: Annular like structure

Br: Branch site

D: Damage

N: Nuclei

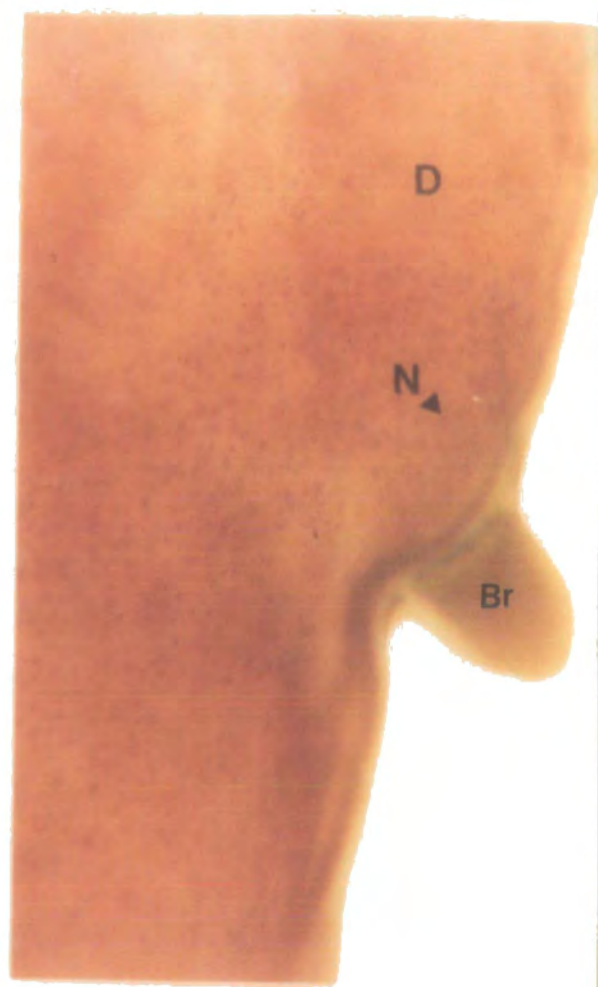
R: Regeneration site

WH: Wound Healing





1



2



3



4

Plate (31)

Distribution of nuclei along the tip segments, during regeneration. Segments were fixed in acetic acid-alcohol and stained with Giemsa

- 1 & 2. Distribution of nuclei during regeneration and formation of septum

1 - magnification X2100

2 - magnification X4000

- 3 & 4. Distribution of stain binding wall substances  
Uvitex BOPT was used and examined under U.v. light

1 - magnification X2600

2 - magnification X2600

A: Apex

Cb: Cell wall brightener

FrN: Fragmented Nuclei

N: Nuclei

RC: Regeneration Channel

WH: Wall Healing or septum formation

UC: Unknown Crystallized body

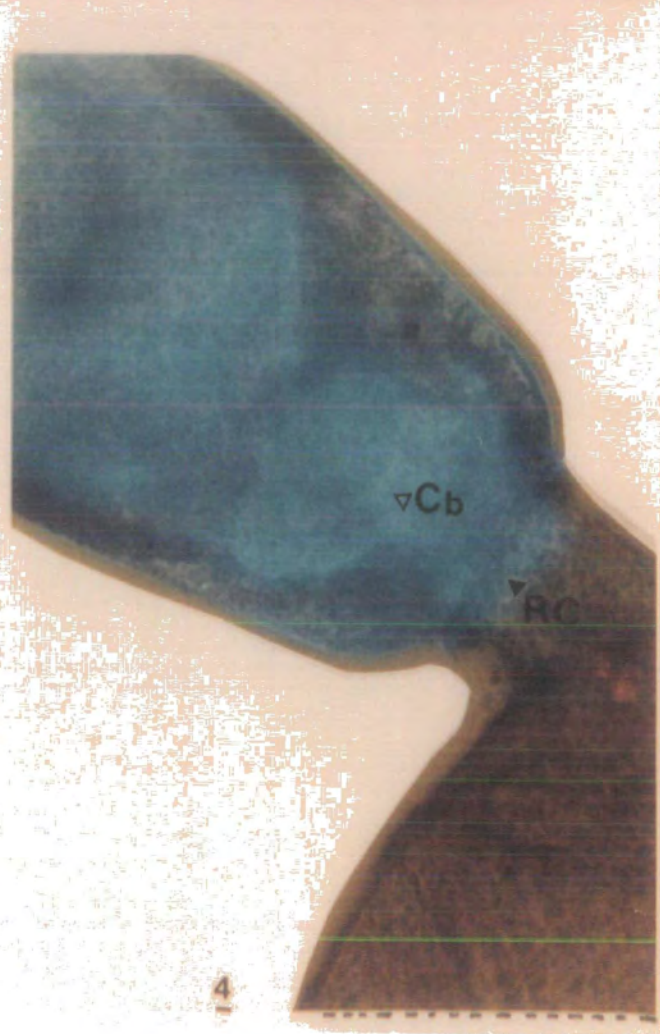
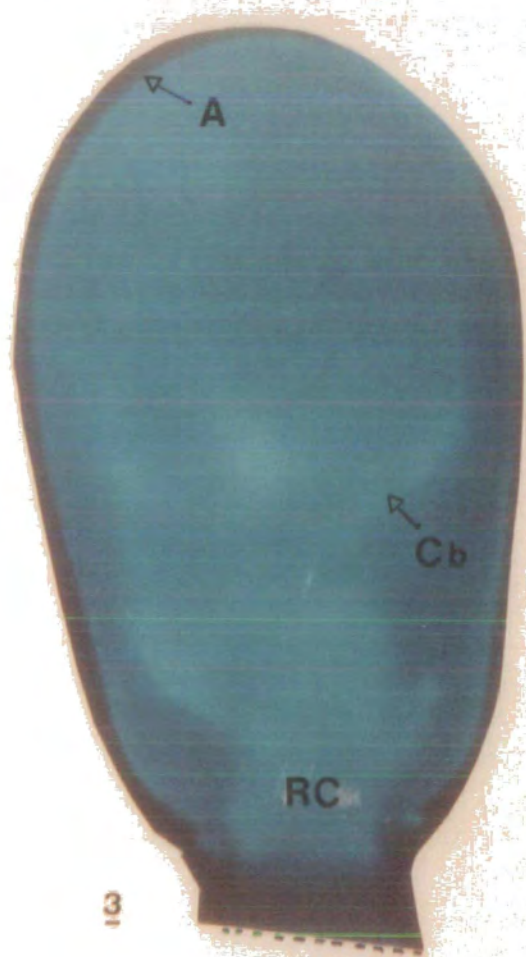
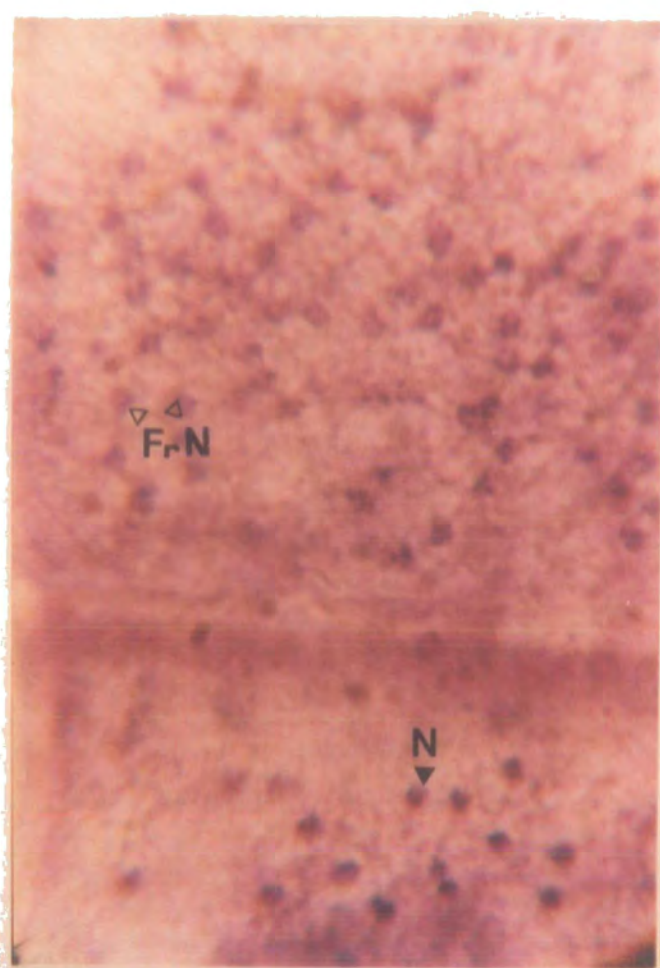


Plate (32)

Distribution of nuclei during regeneration of segments  
Segments were fixed in acetic acid-alcohol

1. Tip segment, stained with Giemsa, showing  
the distribution of nuclei at the cut site

magnification X4000

2. Tip segment, stained with acridine orange, and  
showing more than two developed sporangiophores

magnification X4000

3. Middle segment, stained with Giemsa stain

magnification X2600

4. Basal segment, stained with Giemsa

magnification X2600

A : Accumulation of nuclei

C : Cytoplasm

Cs : Current of strands

N : Nuclei

Rs : Regenerated sporangiophores

S : Sporangiohores

W : Wall or septum formation

U : Unknown body



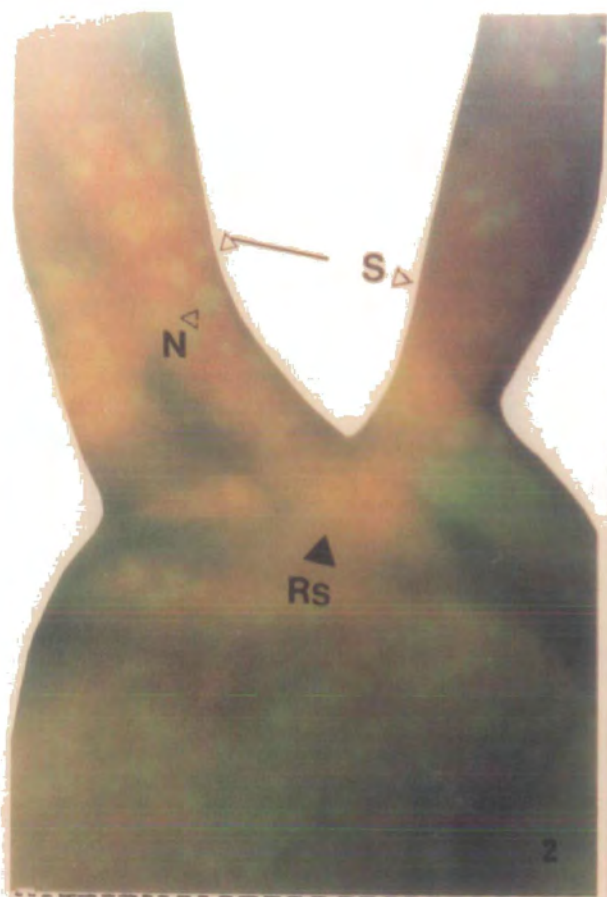
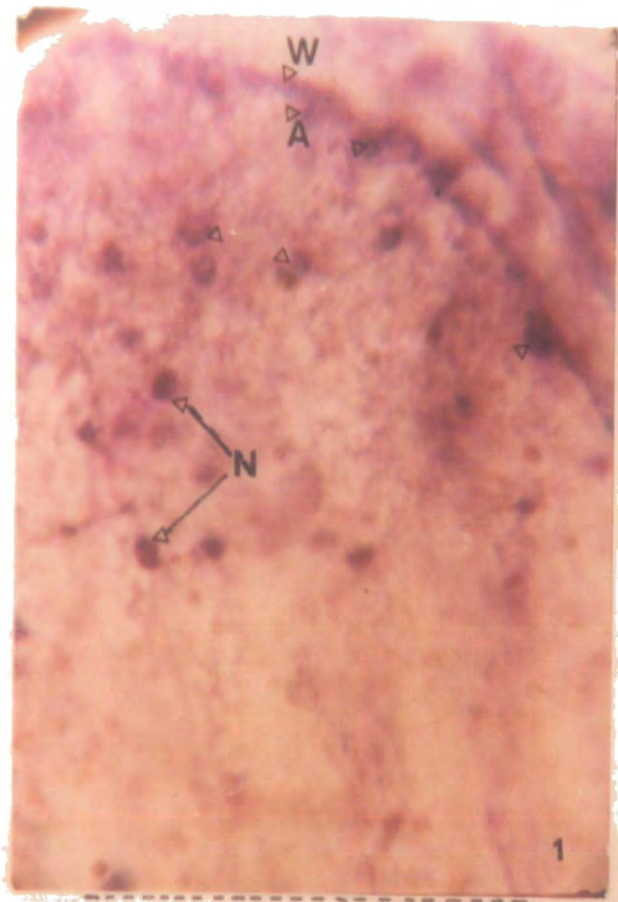


Plate (33)

Distribution of wall staining intensity after regeneration of sporangiophore segments. Uvitex BOPT was used.

1. 12 hr after regeneration of tip segment with cut apex

magnification X1250

2. 12 hr after regeneration of basal segment

magnification X320

3. 12 hr after regeneration of the tip segment with completed apex

magnification X1250

4. 8 hr after regeneration of stump on attached sporangiophore

magnification X1250

5. The tip region of stage II

magnification X52

6. The tip region of stage IV

magnification X41

A: Apex region

B: Basal region

RS: Regenerated Sporangiophore

RB: Regenerated Base

T: Tip region of stump

S: Sporangium



CHAPTER II

Protoplasts Study



## 15. INTRODUCTION

Studies on the cytoplasmic mechanisms of regeneration and development of sporangiophores are hampered by the presence of the cell wall. The penetration of solutes, fixatives, stains and antibiotics is obstructed since the sporangiophore has been shown to have a very thick cuticle as a protective layer (Furch & Gooday, 1978). Therefore it was necessary to develop a system without cell wall i.e. protoplasts, see review by Peberdy (1978). In addition, it was suggested that, studies with the protoplast system may allow a quantitative investigation concerning growth and development of different regions i.e. tip, middle and base, or compartments of the sporangiophore.

Protoplast manipulation has been used as a good model in understanding growth and development of fungi (reviewed by Villaneuva & Garcia-Acha, 1971; Peberdy, 1972; 1978; Peberdy et al., 1976; Vries, 1974). Under stabilizing conditions cultivation of the protoplasts exploits what appears to be an innate capacity to rebuild a cell wall and ultimately develop a form identical to the cell from which they were isolated. Burgeff (1914) showed that when the cut end of one sporangiophore was inserted into the cut end of another,

of different sex, the product would sprout a new sporangiophore. Weide (1939) squeezed the cytoplasmic contents of several sporangia into a common droplet and this droplet was shown to regenerate a hypha when it was in contact with a fragment of wall material. Gruen & Ootaki (1972) developed a method, involving the grafting of two separated segments of sporangiophores from which they concluded that the fused protoplasm showed a regeneration.

Studies on microsurgery, microinjection and heterokaryon production of the cytoplasm (see Bergman et al., 1969; Zalokar, 1969; Ootaki, 1973; Ootaki et al., 1973, 1974, 1977) made use of the fact that the cytoplasmic organelles or components could be separated alive and at a certain function and activity. It has been reported, Bergman et al., (1969), that several methods are possible by which viable protoplasm can be obtained; the tip of the sporangiophore can be cut and the contents allowed to ooze out under positive pressure (analogous to piercing the sporangiophore at the bottom). Also, when the sporangiophore is cut at appropriate levels (analogous to sectioning as a cut made through the vacuolar layer), the cytoplasm can be sucked out with a micropipette. These studies have suggested that a further manipulation of separated cytoplasm may add information on enzyme activity

and wall synthesis.

Protoplasts of Phycomyces blakesleeanus hyphae, of different strains were also isolated by an enzyme, extracted from Trichoderma viride (Binding & Weber, 1974). The above work involved the fusion of the protoplasts to produce a heterokaryon strain. The fusion and regeneration of the cytoplasm of Phycomyces showed (Burgeff, 1914; Weide, 1939; Ootaki & Gruen, 1970) a variance between contents isolated from different regions of hyphae, and it was suggested that these processes may depend on the activity of sub-cellular organelles which were already distributed along the axis (compartmentalization).

It has been found that chitin and chitosan make up the major compositions of the wall of the sporangiophore (Bergman et al., 1969), the possibility that treatment with hydrolytic enzymes could lead a release of the protoplasts in an osmotic stabilized medium, was investigated in this study. In addition mechanical manipulation was also used to isolate protoplasts and to illustrate the interrelationship between location and activity of cellular components, cytoplasmic organelles and components with the cell wall synthesis. Likewise it has been found that an interrelationship exists between the location of cell wall synthesis and the distribution of cytoplasmic organelles

(Weber & Hess, 1976; Grove, 1978).

Regeneration and development of many species of fungal protoplasts has been shown to have two separate stages; production of a new wall and reversion to the final stage of developing a hypha which emerged from the protoplasts (see Gull et al., 1972; Benitez et al., 1975b; Peberdy et al., 1976 and reviewed by Peberdy, 1978).

A pattern of protoplast development in which a budding chain developed was reviewed by Peberdy (1975, 1978) and Vries (1974). One or more normal hypha developed usually from the growing tip distal to the protoplast, as found in Aspergillus nidulans (Gibson & Peberdy, 1972), in Penicillium chrysogenum (Anné et al., 1974) or from the original protoplast as in Trichoderma viride (Benitez et al., 1975a), and in Aspergillus sp. (Davis et al., 1977).

Regeneration of microfibrils of the wall from a granular structure was also shown by McMurrough & Bartnicki-Garcia (1971). Many protoplasts also develop into irregular polymorphic structures without hyphal development. Culture medium and the condition of the protoplasts during the cultivation are reported to have an influence in determining the form and structure of the developed hyphae.

Different methods were used to measure the regeneration and development of the protoplasts. The methods for measurement varied under different conditions of growth and media, Peberdy & Buckley (1973). The method based on the property of adsorption of an optical brightener dye (or fluorescent stain) from a standard solution was recognized to be a satisfactory method for obtaining quantitative measurements of regeneration and development of the protoplasts. Since these dyes are characterized in binding to polysaccharides of the wall (Peberdy, 1978) they may also reveal a real activity of the protoplasts during the regeneration. A method based on counting the protoplasts, may be artifactual in that living and dead structures would be recorded.

Success in any such isolations, manipulations and quantitative measurements of the regeneration and development of the protoplasts of stage I sporangiophore should give a better understanding of the growth and development. Culturing tip protoplasts, which contain active cytoplasmic constituents, could well provide a much better model for an analysis of role of RNA and ribosomes in such development.

## 16. MATERIALS AND METHODS

Synchronized stage I (10-20 mm in length) sporangio-  
phores were plucked carefully from the mycelia and  
manipulated for isolating protoplasts by either of the  
following methods:

### 1. Enzymatic

Protoplasts from sporangiophores at same age and  
same length were isolated, using an enzyme(s) which was  
extracted from the fungus Trichoderma viride by the methods  
of Binding & Weber (1974) and Vries (1974). A Trichoderma  
viride culture was stocked and maintained in the following  
minimal medium where a maximal growth was obtained.

|   |         |
|---|---------|
| D - Glucose                               | 3 gm    |
| $\text{KH}_2\text{PO}_4$                  | 2 gm    |
| $(\text{NH}_4)_2\text{SO}_4$              | 1.4 gm  |
| $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ | 0.3 gm  |
| $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ | 0.3 gm  |
| Mycological peptone                       | 1 gm    |
| Urea                                      | 0.3 gm  |
| Pure glycerine                            | 10 ml   |
| Distilled water                           | 1000 ml |

Solid medium was prepared by adding 20 g.l<sup>-1</sup> agar to  
the above medium. Media were autoclaved in a similar way  
as for Phycomyces. The culture was routinely recultured  
every week and cultures were incubated under continuous

fitted head illumination of white fluorescent light (20W) in a growth chamber, see Plate(1(1)) and at room temperature  $23 \pm 2^{\circ}\text{C}$ .

1. Preparation of the wall lysing enzyme(s)

1 ml of a spore suspension of Trichoderma viride (approximately  $10^5$  spores) was inoculated into a 2L flask containing 250 ml liquid medium, with glycerine omitted. The autoclaved medium contained 5 gm  $\cdot \text{L}^{-1}$  of enzyme substrates, which were dried sterilized Phycomyces sporangiophore wall (see below) or commercial chitosan.

2. Preparation of the walls of sporangiophores as a substrate for activating secretion of lytic enzyme(s)

Sporangiophores grown in minimal medium were harvested and ground after freezing in liquid nitrogen. Grinding was carried out using a pestle and mortar. After grinding, the extracts were washed several times with distilled water, until no more cytoplasm was removed. Each washing step was followed by centrifugation at  $500 \times g$  (MSE Bench centrifuge) for 5 minutes. The sedimented walls were collected and dried in an oven at a temperature of  $55^{\circ}\text{C}$  for 2-5 minutes. This procedure was repeated until enough weight was obtained.

Cultures were then maintained at  $23 \pm 2^{\circ}\text{C}$  under 20W lamps on an orbital shaker at 120 revs/min. Mycelia

which were developed after a period of 2-3 days were filtered off and the culture filtrate (liquid) was retained to extract the lytic enzyme(s). Ammonium sulphate was added to the supernatant to 80% saturation.

The preparation was kept for 4°C for 24 hours for complete precipitation, following this time, it was centrifuged at 14000 x g (MSE 18 centrifuge) for 8 minutes. The precipitate was collected and dissolved in a minimal volume of 10<sup>-2</sup>M phosphate buffer at pH 5.8 and the supernatant was discarded. The buffered enzyme solution was dialysed against water for a further purification. Any precipitation obtained in this step was discarded, and the buffered solution of lytic enzymes was lyophilized and kept at -20°C until needed.

The concentration of protein was estimated by Lowry et al., (1951) method which was described in materials and methods, Chapter I. 1-3 mg.ml<sup>-1</sup> protein was estimated, and as a preliminary test 1 mg.ml<sup>-1</sup> protein was found to have the ability to lyse the wall, the protoplasts were liberated from the tip region, Figure(20(1)). Stability of the protoplasts during the incubation was shown to vary within the reaction. The optimal condition was obtained by testing the reaction under several points.



3. Effect of the osmotic stabilizer of the lytic medium on the isolation and stability of protoplasts

Prior to manipulation for isolation protoplast, sporangiophores were washed in 0.05M phosphate buffer pH 7.0 to remove any spores present. 1gm fresh weight of stage I sporangiophores were incubated with 1-3 mg·ml<sup>-1</sup> wall lysis enzyme(s) in a closed tube at 23 ± 2°C. Different osmotic stabilizers were included NaCl, KCl, MgCl<sub>2</sub>·6H<sub>2</sub>O, Sorbitol, MgSO<sub>4</sub>·7H<sub>2</sub>O, NH<sub>4</sub>Cl, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, KH<sub>2</sub>PO<sub>4</sub>. Initially different molarities (0.1-1M) for each compound were tested and by examination, it was found that more than 0.8-1M, produced extensive lysis and the protoplasts also showed plasmolysis. This condition was less in sorbitol and MgSO<sub>4</sub>·7H<sub>2</sub>O. Additionally 0.5M sorbitol was shown to give contamination with yeast and other organisms. Further studies showed that MgSO<sub>4</sub>·7H<sub>2</sub>O at 0.5M appeared to achieve a better stabilization and a good yield of protoplasts, Figure (20(2)).

4. Effect of pH of lytic medium on the stabilization of protoplasts

0.05M phosphate buffer was shown initially to achieve a better stabilization as judged by microscopy. The final yield of the protoplast was shown to vary at different pH values.

As shown, a greater yield of protoplasts was obtained in lytic medium containing  $2 \text{ mg.ml}^{-1}$  enzyme with  $0.5\text{M}$   $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  and at  $0.05\text{M}$  phosphate buffer of pH 5.8-6.0, Figure (20(3)). This lytic medium was used as a standard in the experimental sets of the protoplast studies. Protoplasts were counted using a hemocytometer and the methods are described below.

##### 5. Preparation of protoplasts for counting and regeneration studies

An optimal yield of stabilized protoplasts was found to be achieved after 3-4 hours of incubating the sporangiophores (i.e. 1 gm sporangiophore fresh weight per 20 ml of lytic medium). After liberating all of the protoplasm from the incubated sporangiophores (examined under the microscope), the incubation mixture was centrifuged in an MSE bench centrifuge at a speed of  $500 \times g$  for 10 minutes. This resulted in a layer of vacuolated protoplasts floating at the meniscus, free of any residues of sporangiophores. The floating protoplasts were pipetted into a graduated tube. The sedimented pellet, contained the residues of sporangiophores and also the non-vacuolated protoplasts. Using a fine and sterilized sharp needle the residual sporangiophores were removed and the pellet of sedimented protoplasts left. The supernatant was added to the tube containing the

Figure (20)

- 1 - Effect of different concentrations of enzyme(s) on the release of protoplasts
- 2 - Effect of different osmotic stabilizers on the release of protoplasts in the presence of  $2 \text{ mg.ml}^{-1}$  enzyme(s)

□  $0.5 \text{ M MgSO}_4 \cdot 7\text{H}_2\text{O}$

○  $0.5 \text{ M sorbitol}$

△  $0.5 \text{ M MgCl}_2 \cdot 6\text{H}_2\text{O}$

○  $0.5 \text{ M NH}_4\text{Cl}$

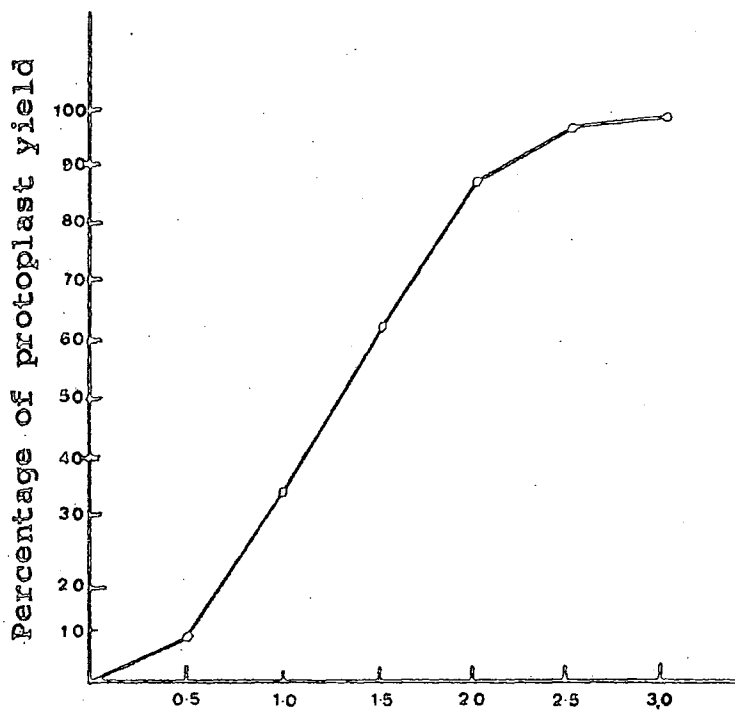
○  $0.5 \text{ M (NH}_4)_2\text{SO}_4$

△  $0.5 \text{ M KH}_2\text{PO}_4$

▽  $0.5 \text{ M NaCl}$

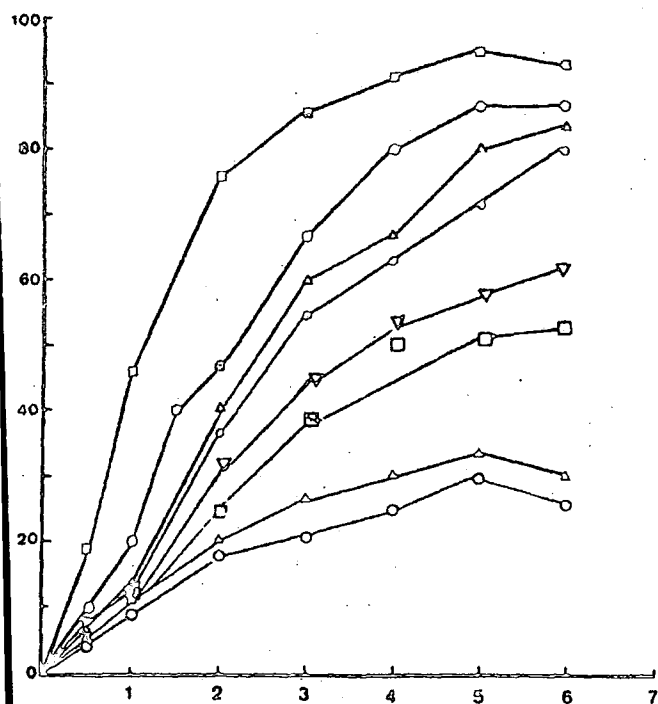
□  $0.5 \text{ M KCl}$

- 3 - Effect of different pH values of  $0.05 \text{ M}$  phosphate buffer on the release of protoplasts in lytic medium contained  $0.5 \text{ M MgSO}_4 \cdot 7\text{H}_2\text{O}$  and  $2 \text{ mg.ml}^{-1}$  enzyme(s)



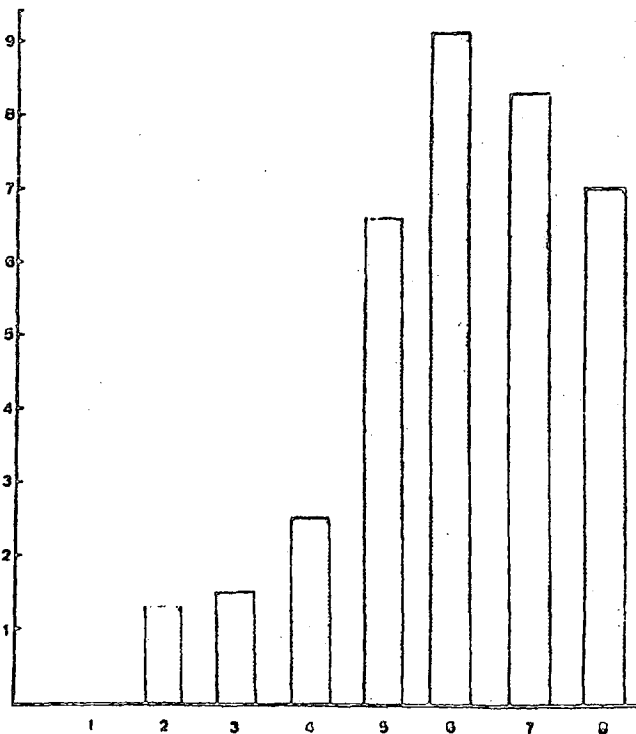
Concentration of the enzyme mg.ml<sup>-1</sup>

1



Time (hrs)

2



pH

3

vacuolated protoplasts. The sedimented protoplasts were washed with 3 ml of 0.5M  $\text{MgSO}_4$  at pH 6.0 in 0.05M phosphate buffer. This was repeated three times to remove any enzyme excess. The pellet was then resuspended in a known volume of the stabilizing solution and this was used for counting and regeneration studies. The vacuolated protoplast suspension was filtered using a suitable micro-paper; this was carried out after measuring the diameter of the protoplasts. Filtration was followed by washing the vacuolated protoplasts and this treatment was also repeated three times to remove enzyme excess. Vacuolated protoplasts then were resuspended in a known volume of stabilizing solution and this was used for counting and regeneration studies. The relative proportion and total numbers of vacuolated and non-vacuolated protoplasts are shown in Figure (21).

#### 6. Methods for counting and examining protoplasts

Protoplasts were counted using a hemocytometer system which is described by Hall & Hawkins (1975), Parsons & Schapiro (1975). Suspension of protoplasts were counted until a constant value was obtained for the sample. By correction for volume, the total number of protoplasts in a given extraction was determined.

Figure (21)

Protoplasts isolated by enzyme(s), from sporangiophore. 0.5 gm in fresh weight, 15-20 mm in length and at different intervals. Sporangiophores were submerged in lytic medium containing  $2 \text{ mg.ml}^{-1}$  enzyme(s) with 0.5 M  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  and at pH 6.5 phosphate buffer



Non-vacuolated protoplasts

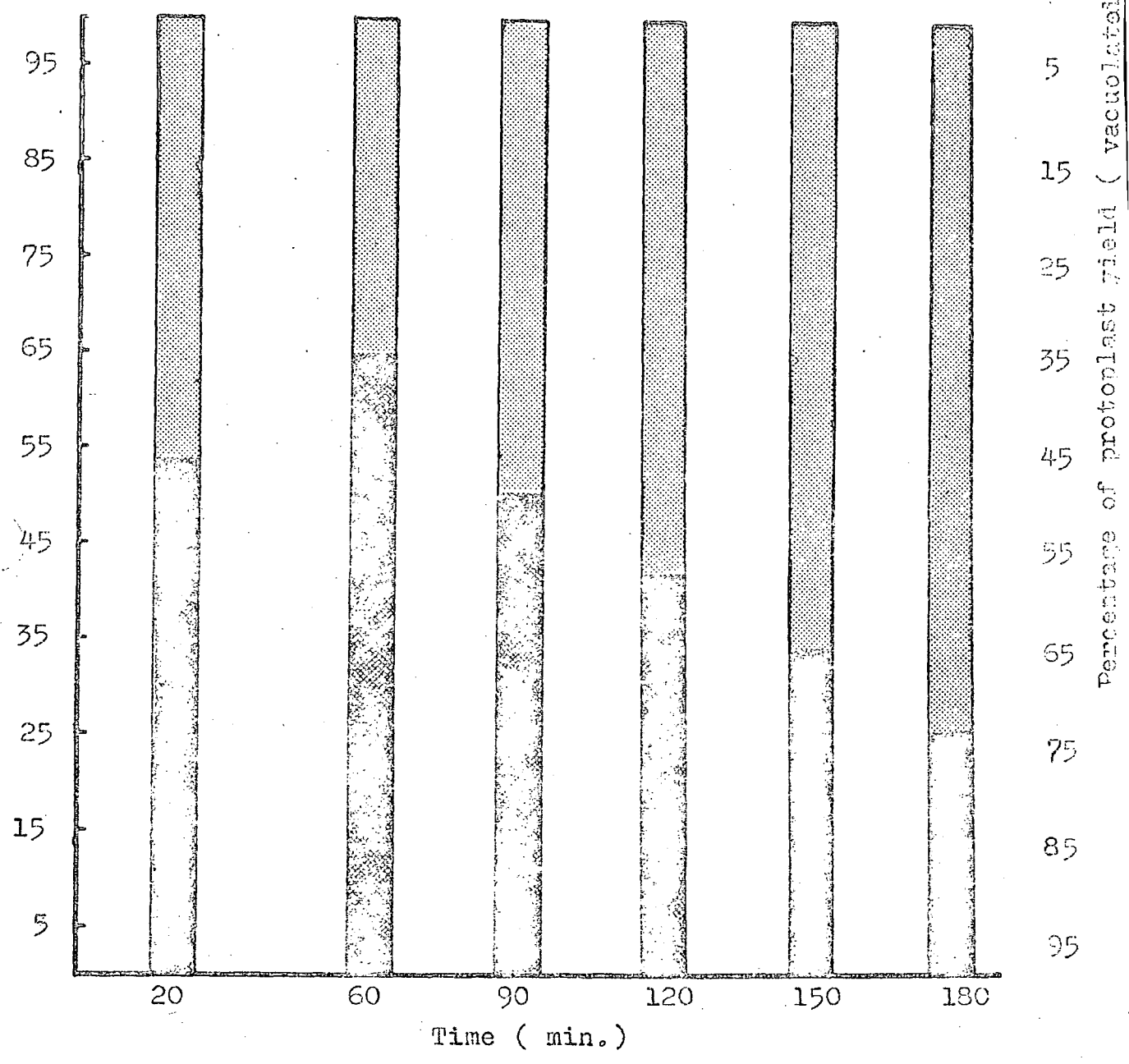


Vacuolated protoplasts

(Percentage of protoplast yield (vacuolated))

Total number of protoplasts

$18 \times 10^7$        $20 \times 10^7$        $8 \times 10^7$        $6 \times 10^7$        $5 \times 10^7$        $4 \times 10^7$



## 2. Mechanical

The protoplasm of sporangiophores was also isolated by placing submerged sporangiophores in a hypertonic solution of  $1M \text{MgSO}_4 \cdot 7H_2O$  in phosphate buffer pH 6.0. This led to extrusion of protoplasm from the cut ends of isolated sporangiophores. Highly plasmolysed protoplasts were formed during this method, which could be due to the higher concentration of the solution, but protoplasts were seen to have recovered when they were transferred to a solution of  $0.5M \text{MgSO}_4 \cdot 7H_2O$  in phosphate buffer pH 6.0.

An alternative method which was employed involved isolating protoplasts from full length sporangiophores or from sporangiophores cut into segments (equal in length) by placing them submerged in the osmotic stabilizing solution ( $0.5M \text{MgSO}_4 \cdot 7H_2O$  in phosphate buffer pH 6.0). Then, when either end of each segment was pierced, protoplasm was seen to be released in a condition similar to that observed in the enzymatic system. Piercing gently at the apex of full length sporangiophores, with a pair of very fine and sterilized forceps, was also found to release the protoplasmic contents from the basal cut end of these sporangiophores. No wall residues were seen associated with the protoplasm, thus it was suggested that these aggregates of protoplasm may represent protoplasts. Counting and examination were carried out in a



similar way as described for protoplasts released by an enzyme(s) effect.

### 3. Regeneration medium

Medium which was used for achieving a maximal growth of sporangiophore was also used as a regeneration medium for protoplasts, but included  $0.5M \text{ MgSO}_4 \cdot 7\text{H}_2\text{O}$  pH 6.0 in  $0.05M$  phosphate buffer.

The effect of number of protoplasts and medium ratio in obtaining an optimal regeneration was tested. An optimal regeneration (producing a wall) was examined by measuring the amount of stain (Uvitex BOPT) adsorbed by the protoplasts at 12 hours after incubation, Figure (22).

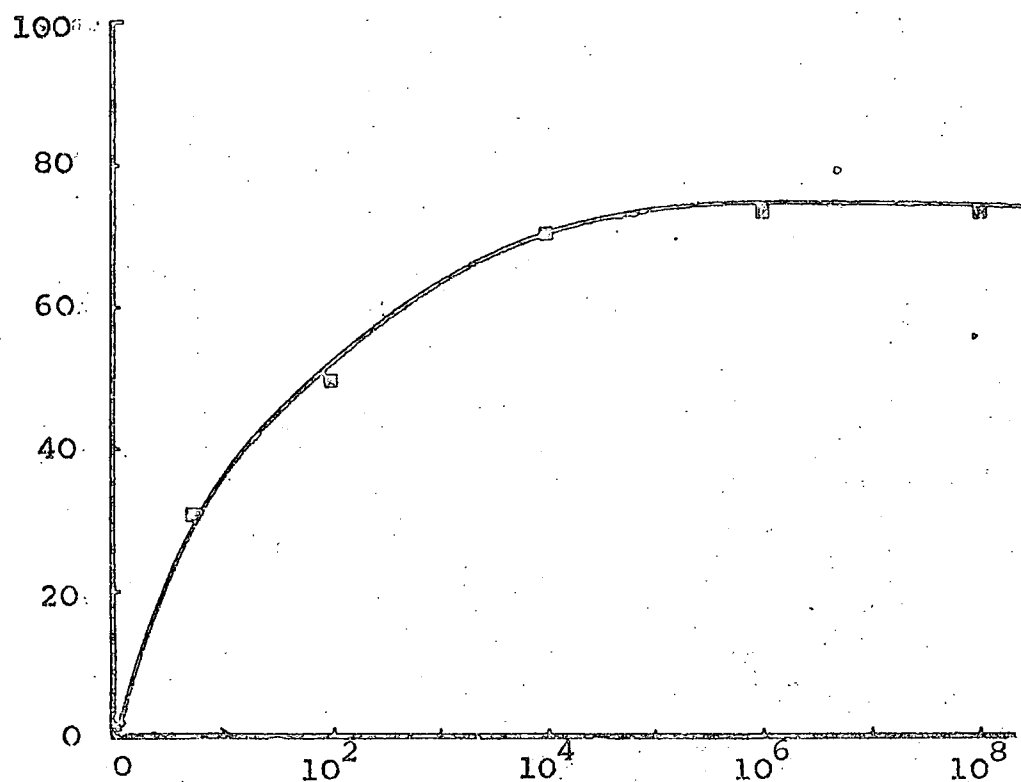
Optimal conditions for achieving a maximal adsorption of stain are described in material and methods of Chapter I. It was found that a sharp increase of stain adsorption appeared up to  $10^6$  protoplasts  $\cdot \text{ml}^{-1}$  regeneration medium, Figure (22), but further increase of protoplast numbers did not give an increase in the stain adsorption.  $10^6$  protoplasts  $\cdot \text{ml}^{-1}$  regeneration medium were routinely used as a standard in the study of regeneration of protoplasts of different regions of sporangiophores.

As a preliminary test for culturing the protoplasts, semi-solid medium was used and prepared by adding 10 gm of agar to 1L of liquid regeneration medium. The effects

Figure (22)

The effect of concentration of protoplasts on  
regeneration

Uvitex BOPT adsorbed  $\mu\text{g} \cdot \text{ml}^{-1}$



Number of protoplast  $\cdot \text{ml}^{-1}$  regeneration medium

of the regeneration media on the development of the protoplasts are described in the results sections.

4. Methods for determining the regeneration and growth of protoplasts

Three main methods were used here as outlined below. Increase in dry weight with time was determined as a preliminary test for growth. This was carried out by drying the protoplasts at 55°C for 2-5 minutes. Limitations to this method were encountered with small quantities of protoplasts.

Additionally the diameter of the protoplasts and/or the length of the regenerated hyphae were measured. Limitations to these methods were also encountered since the basal protoplasts were shown to have diameters larger than those of protoplasts derived from the tip region; since tip protoplasts produced new cell wall quicker than basal protoplasts, there was an earlier limitation to expansion. Thus it is suggested that increases in the diameter of the basal protoplast could be due to the penetration of solutes with less restriction due to wall surrounding the protoplast. Additionally length measurements of the regenerated hyphae were shown unsuitable because hyphae clumped and made measurements difficult.

Measuring the amount of regenerated wall produced by protoplasts during development was found to be the most suitable method for obtaining an accurate and real measurement for the viability of protoplasts to regenerate and to produce hyphae (see also Peberdy & Buckley, 1973; Peberdy, 1978). This method proved a good system for determining quantitatively the location of active wall synthesis throughout the sporangiophore. The amount of the regenerated wall of protoplasts was determined by measuring the amount of fluorescent stain (Uvitex BOPT) adsorbed, as described previously in Chapter I.

##### 5. Cytological and biochemical observations: Preparation of protoplasts for staining and extraction

Isolated and regenerating protoplasts were centrifuged at 500 x g (MSE centrifuge) for 5-10 minutes. They were washed three times with 0.05M phosphate buffer pH 6.0. Before staining they were fixed in acetic acid-alcohol (1:3 v/v) for 30 minutes, then they were washed in phosphate buffer for 15 minutes.

Staining was carried out in test tubes, and each treatment of fixation, washing and staining was followed by centrifugation at 500 x g for 5 minutes for a complete sedimentation, as this appeared to be easier for manipulation. D.P.X. was usually used as a mountant.

Coverslips smeared with a layer of solid medium were used to cultivate protoplasts in examining the morphological change during regeneration. This technique minimized mechanical damage during the handling of protoplasts. Coverslips carrying the regenerating and developing protoplasts were placed on slides after staining. Nuclei were stained with acridine orange, Giemsa and Feulgen stains as previously described. Location of wall during regeneration was determined microscopically using Uvitex BOPT stain (0.1% solution in 0.1M phosphate buffer at pH 6.5 and at 30°C in conjunction with u.v. microscopy.

RND, DNA and protein were extracted by a method also described in Chapter I, materials and methods.

CHAPTER II

Results

## 17. GENERAL OBSERVATIONS

### 1. Isolation and Characterization

#### 1. Enzymatic release

Through the action of the enzyme(s) of Trichoderma viride protoplasmic spheres were released from sporangio-  
phores of Phycomyces blakesleeana. It was observed,  
that these protoplasmic spheres emerged through ruptures  
in the cell wall leaving empty cylindrical sporangiophore  
wall behind. It appeared that spheres were lacking in a  
rigid cell wall and that they were osmotically sensitive.  
According to Villanueva & Garcia-Acha (1971), Peberdy  
et al., (1976) and Peberdy (1978), these structures can  
be considered as protoplasts.

Emergence of protoplasts initially was from a pore  
at the apex region of the sporangiophore and lysis of the  
wall of that region could be the reason. A similar  
enzyme system was used by Binding & Weber (1974) in  
isolating mycelial protoplasts of Phycomyces.

Emergence through the pore, initially, of a non-  
vacuolated structure followed by vacuolated structure  
was taken as evidence that true protoplasts were being  
released from the sporangiophore in a sequential  
pattern.



Isolation of the cytoplasm of the sporangiophore was achieved using a suitable lysis medium, (see materials and methods) and in the presence of 0.5M  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  as a suitable osmotic stabilizer. Use of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  as an osmotic stabilizer was also recommended by Gascon & Villanueva, (1965), Sietsma et al., (1967), Sietsma & Wouters (1973), Dooijewaard-Kloosterziel et al., (1973), Vries (1974).

The actual cutting of the sporangiophore allowed some leakage of the protoplasm from the base, but this stopped when protoplasts emerged from the tip region of the sporangiophore. The cytoplasm released from the tip region emerged to form small dense spherical protoplasts free of the parent sporangiophore wall. During the formation of protoplasts, sporangiophore contents were continuously displaced towards the pore. The extrusion of vacuolated protoplasts and the widening of the pore and rupture of the soft wall were observed. In some cases part of the end of the sporangiophore was locally plugged with the large vacuole present in cytoplasm coming from the base. Constriction of the cytoplasm into smaller protoplasts was observed during the process of incubation and subsequent vacuolation.

Time and diameter of the protoplast released from the sporangiophore are shown in Table (5).

Table (5)

Time, diameter and characteristics of protoplasts released from sporangiophores.

| <u>Time (hour)</u> | <u>Diameter (<math>\mu</math>m)</u> | <u>Characterizations</u>   |
|--------------------|-------------------------------------|--|
| 0-1                | 4.1-5.5                             | Non vacuolated, dense granular cytoplasm and containing small vacuoles, 1-3 nuclei per protoplast. |
| 1-2                | 4.1-8.25                            | Vacuolated, cytoplasm diffusely granular, large vacuoles absent, 4-8 nuclei per protoplast.        |
| 2-3                | 4.1-13.75                           | Larger vacuole, less particles<br>1-2 nuclei per protoplast  |

The amounts of vacuolated and non-vacuolated protoplasts released during the treatment are shown in Figure (21). Non-vacuolated protoplasts were originally released from the upper part of sporangiophores, while the vacuolated protoplasts were attributed to the parts below the tip region (see Chapter I).

## 2. Mechanical release

Protoplasts were also squeezed out from different segments, after placing them in osmotically stabilizing medium (see materials and methods). When segments were used both vacuolated and non-vacuolated protoplasts emerged from the cut end and were counted, and the results are shown in Figures (23 and 24). However, segments from the tip region gave more protoplasts than the middle and basal segments. It was also seen that non-vacuolated protoplasts were released from the tip region than from parts below the tip region. Characterization of the protoplasts appeared to be similar to those described above.

Different initial length sporangiophores of stage I were manipulated for protoplasts isolation, using the enzyme(s) system, see Figure (25). Total numbers of protoplasts (vacuolated and non-vacuolated) were determined and it was found that there was an increase in numbers released as the length of the sporangiophore was increased. However,

Figure (23)

Total numbers of protoplasts, isolated from tip, middle and basal segments. Segments were equal in length and for isolation, they were submerged in an osmotic stabilised media ( $0.5 \text{ M MgSO}_4 \cdot 7\text{H}_2\text{O}$  and at pH 6.5). Procedures of mechanical isolation were used

A - Apical

M - Middle

B - Basal

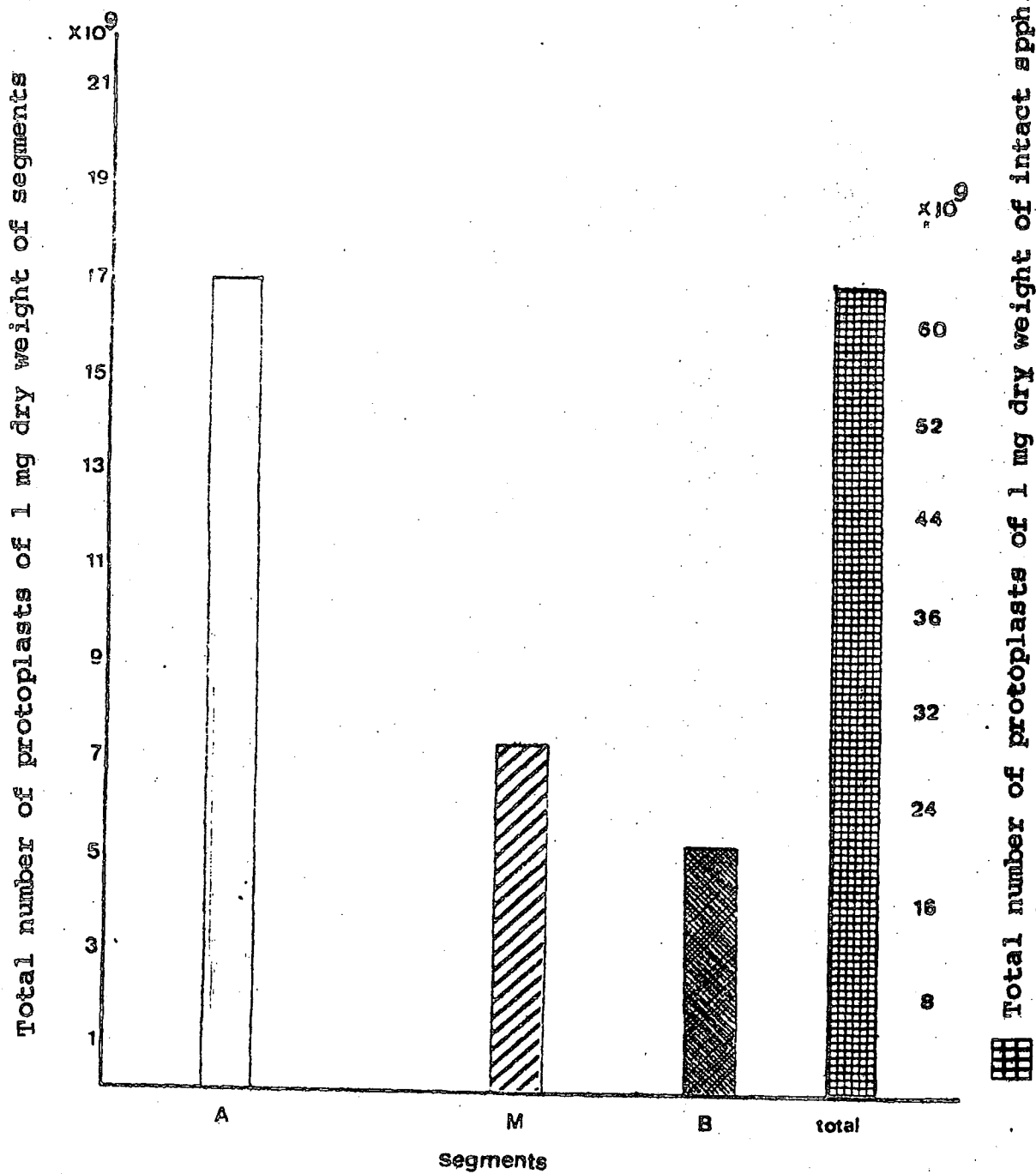


Figure (24)

Total numbers of protoplasts isolated mechanically from segments of stage I sporangiophore, and from full length sporangiophores (20 mm). Sporangiophores, in segments, or full length, were submerged in osmotic stabilized medium and protoplasts were squeezed by piercing one end of the sporangiophore

A Protoplasts of tip segments, 4 mm in lengths

^  
A Protoplasts of tip segments, 4 mm in lengths  
(with tapered apex removed)

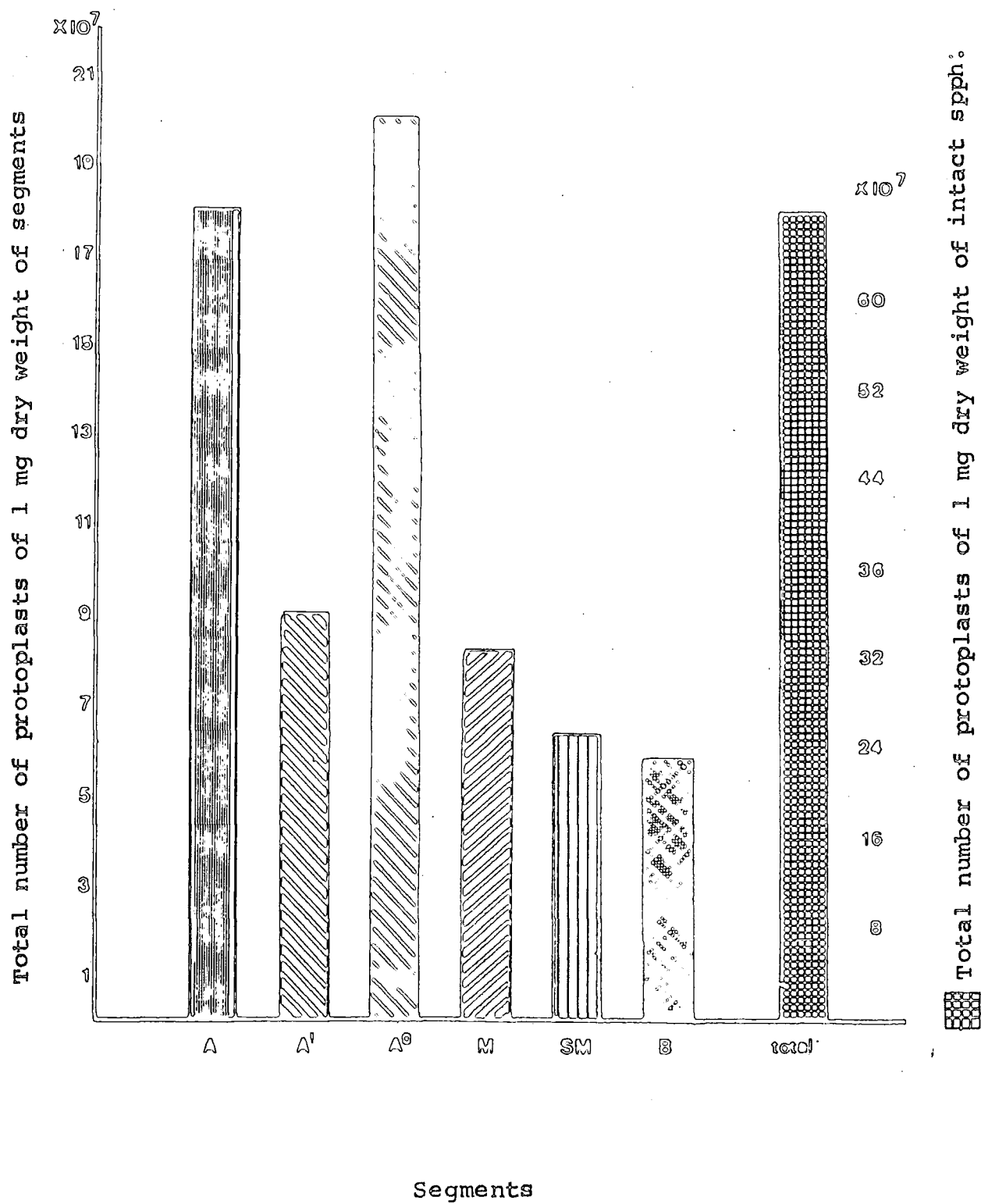
°  
A Protoplasts of segments below the tip  
region, 4 mm in lengths

M Protoplasts of middle segments, 4 mm in  
lengths

SM Protoplasts of Sub-Middle segments, 4 mm  
in lengths

B Protoplasts of basal segments, 4 mm in  
lengths

Total protoplasts number isolated from  
sporangiophores full lengths



after 40-60 mm, the total number decreased. These observations are also in agreement with an optimal dry weight and this corresponds with the high level of cellular components.

The influence of culture age at harvesting, on the protoplasts yield, could be explained by the fact that the activity of the endogenous wall lytic enzyme, possibly used by the fungus in wall assembly, is highest in the younger zones. Therefore there is an enhancement of the lytic effect by the exogenous enzyme(s), Bartnicki-Garcia & Lippman (1972). It may also be the case that the highest yield of the protoplasts from the younger sporangio-  
phore, and also from the younger zone, tip, may be attributed to the greater susceptibility of the wall to undergo lysis when compared with the more differentiated wall of the older sporangio-  
phore or older region, base. The wall composition possibly plays a part during lysis since the presence of e.g. melanin and sporopollenin at parts below the tip region of the sporangio-  
phore, have been found to act as protective agent (Furch & Gooday, 1978). Additionally, melanin-bound chitin in Aspergillus nidulans was found to be resistant to enzymic degradation (Bull, 1970).



The quantity of the Uvitex BOPT for wall staining was found to be associated with the age and numbers of protoplasts, Figure (25). The older region of sporangio-phores also acquires less wall staining than the younger region (Chapter I), indicating a more stable wall structure, which is probably less susceptible to degradation.

## 2. Culture and development of protoplasts

When suspended in an osmotically stabilized growth medium (see materials and methods) some of the protoplasts showed the capacity to return to the normal hyphal form of the fungus. Success of the measurements of the development were shown to depend on the culturing methods being used. Differences in the morphological structure of the regenerating protoplasts were also observed when using different culturing methods. Initially use was made of solid medium (see materials and methods), for counting numbers of the regenerated colonies from protoplasts. This method, however, was not suitable since protoplasts developed into irregular polymorphics structure with random production of hyphae, Plates (34(2,4,5,6), 35 (1,2)), whereas others showed no development of hyphae, Plates (34(3)). Developed hyphae were also shown to give clumps which made accurate counting impossible, Plates (34(3,4) , 36)).

Figure (25)

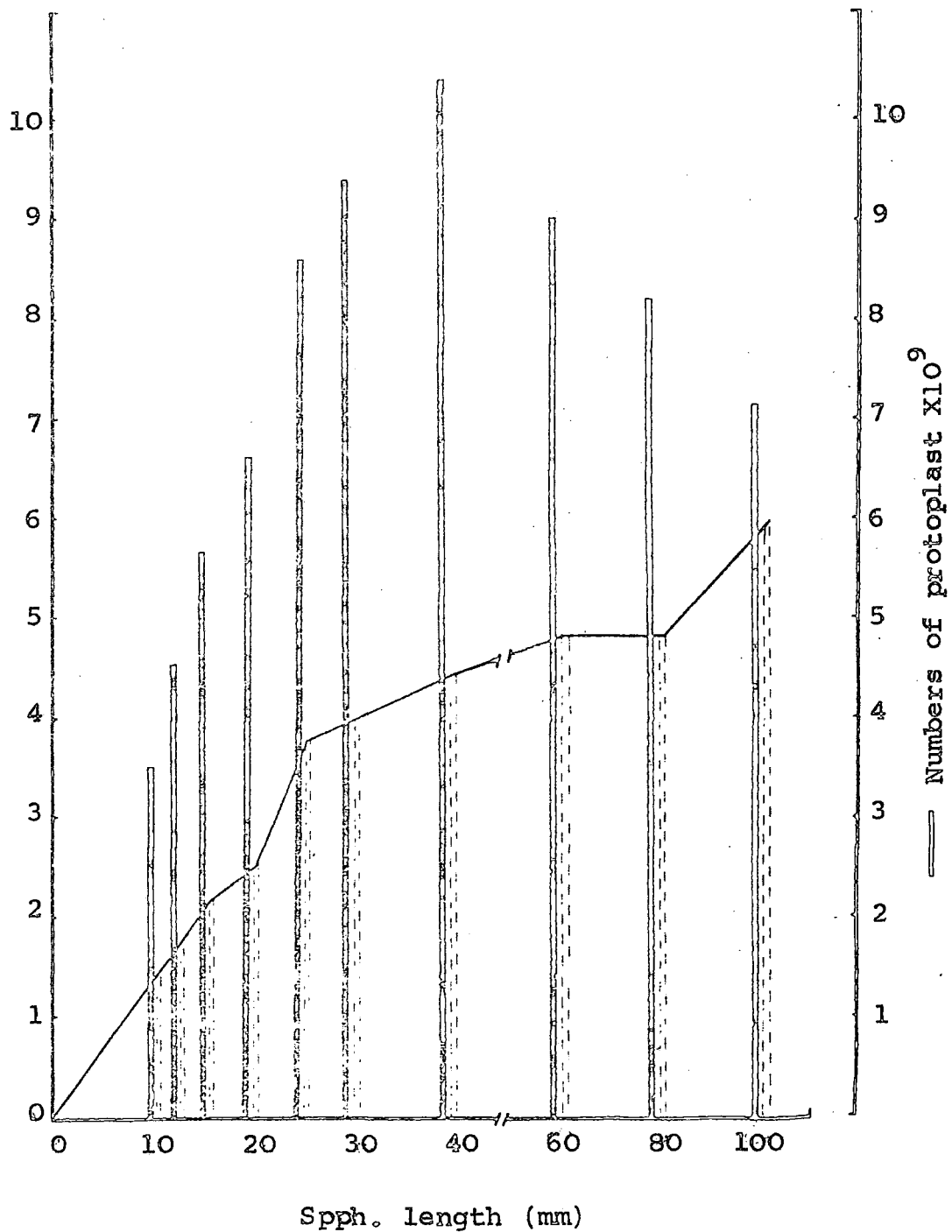
Total numbers of protoplasts released from sporangiophores of different initial lengths. The amount of Uvitex BOPT by the wall of sporangiophore at each length is also shown.

The solid lines represent the total number of protoplast of intact sporangiophore.

The dotted lines represent the amount of Uvitex BOPT adsorbed by the wall of the sporangiophore (1 mg dry weight wall per 5 ml standard stain solution were incubated in the dark for 4 minutes).

The amount of stains adsorbed by the wall were calculated from the calibration curve in Figure (2).

Uvitex BOPT concentration  $\mu\text{g} \cdot \text{ml}^{-1} \times 10$



Development of hyphae from protoplasts on the solid medium were shown to be similar to those developed during spore germination. During development of hyphae from protoplasts, foot cells were also identified. Sporangioophores also appeared to be initiated from the foot cells, Plates (35(5), 37(1)). Foot cells were usually found at the centre of the colony.

Different regions of maturation of hyphae developing from protoplasts were recognized. The peripheral or marginal region showed to be characterized with the presence of tips of leading hyphae, Plate (37(1,2)). The central mature region showed to contain foot cells producing sporangioophores and between these two regions there was a region containing fewer and smaller foot cells compared with those seen in the central region. Additionally, the central region contain greater concentration of RNA than the marginal region, Plate (36(1,2)). These observations can be considered as evidence supporting the previous results for the requirement of foot cell in sporangioophore growth (Chapter I).

Different patterns of development were also identified when protoplasts were grown in liquid medium. One or more hyphae were usually seen to arise directly from the protoplast and grew to form a colony. During incubation of

regenerating protoplasts, submerged in the semi-solid and liquid medium, the developed hyphae showed to give an aberrant pattern of budding chains, see Plates (39(3), 42(2,3,4)). This was also seen by constricting the normal hyphae into cells with different shapes, Plate (3).

During culturing the protoplasts in a shallow layer of liquid regeneration medium, no such observations were observed, Plate (39). Limited oxygen in the former medium could be attributed to the aberrant development. The composition of the wall formed by the protoplasts may also have played a role in determining the form of development. In liquid medium another aberrant pattern of development was also identified, when the protoplasts bulged out on one side, developing a bud like structure, Plate (40(1,4)). Also protoplasts were seen to aggregate in groups consisting of 2-3 protoplasts, Plate (40(2)).

Aggregation of protoplasts were shown sometimes to produce a thick wall like structure, Plates (41,42 (1)). Individual protoplasts were shown also to be surrounded by a wall before hypha development began and the wall gradually thickened. To show this, protoplasts after regeneration for 3-4 hours were transferred to water. Under these conditions bursting of the protoplasts were seen but only at the selected sites on the wall, Plate (40(5)). The

parts which did not burst were suspected of being the location of hyphal development.

Generally, when culturing protoplasts on solid and semi-solid, Plate (40(3)), some difficulties were encountered in obtaining accurate quantitative measurements of regeneration, since irregular aggregates of regenerated hyphae on solid medium, and presence of more than one developed structure on semi-solid medium, both creating difficulties in measuring the length and diameter of the regenerated protoplasts. Different patterns of protoplast regeneration and development were also observed by many investigators using different methods of manipulation and culturing (Bachmann & Bonner, 1959; Garcia-Acha et al., 1966; Fuki et al., 1969; Peberdy & Gibson, 1971; Villaneuva & Garcia-Acha, 1971; Peberdy, 1972, 1978; Gull et al., 1972; Sietsma & De Boer, 1973; Sietsma & Wouter, 1973; Dooijewaard-Kloosterziel, et al., 1973; Anne' et al., 1974; Benitez et al., 1975a,b) who found different frequencies of regeneration of the same protoplast population in agar medium and in liquid medium.

Incubation of the protoplasts in a shallow layer of osmotically stabilized liquid medium for regeneration was shown to be more suitable for obtaining a good regeneration, and normal development of hyphae, and thus was used in the

further studies on protoplasts. However, for RNA, DNA, protein and ribosomes extraction, protoplasts were grown in a shallow liquid medium prior to plating onto solid medium. Aeration of the pre-incubated liquid medium protoplasts, activated the synchronization of the developed hyphae. This observation is similar to that seen previously for normal cultures where a nearly synchronized sporangio-phore formation was obtained when the culture was subjected to sudden aeration.

18. INFLUENCE OF SITE OF ORIGIN OF PROTOPLASTS, ON  
REGENERATION AND DEVELOPMENT

Protoplasts isolated from different regions of stage I sporangiophores were investigated for their ability to regenerate and grow. Synchronized stage I sporangiophores were cut into segments which were equal in length (3-5 mm). The activity of protoplasts to regenerate a new wall was tested quantitatively using the method of Uvitex BOPT brightener (see materials and methods of Chapter I).

More protoplasts were released from the tip region of the sporangiophore than from an equal length of middle and base regions, Figure (23). When a sporangiophore was cut into five equal length segments, greater numbers of total protoplasts were released from the upper part, see Figure (24). The increase in the number of protoplasts from the tip was balanced by their being smaller than those from sections lower down. The presence of more cellular components in the cytoplasm in the tip of the intact sporangiophore could allow the fragmentation into many discrete protoplasts. In contrast, 2-3 mm long segments in length of the extreme apex released less protoplasts than the sub-apical segment. Possible explanations for these observations are that there is a lack of organelles in the extreme apex region of sporangiophore (as found in Chapter I) or because of the tapered tip there is a greater



cell wall/protoplast ratio.

Regeneration of protoplasts from various sporangio-  
phore was examined using similar regeneration medium  
described above. The activity of protoplasts of tip,  
middle and base segments to regenerate and produced a  
new cell wall was measured at intervals, Table (6). More  
adsorbtion of stain was shown to occur on to the  
protoplasts and could be attributed to a greater new wall  
production.

Protoplasts which were released from the tip segments  
were seen to produce a thick wall around themselves as an  
individual unit. However, protoplasts which were released  
from the middle and basal segment showed aggregation into  
linear groups, Plates (42(2,3,4), 43(3,4)) and also Table (7).  
This may be attributed to the fact that the tip protoplast  
is self sufficient for new wall production, because it  
contains more substance(s) or organelles which are  
required for this process. Aggregation of the middle and  
basal protoplasts in a group could be interpreted as their  
requirements to achieve sufficient amount of organelles  
for producing a new wall, since each protoplast may not  
be self sufficient. Tip protoplasts also formed linear  
aggregations but usually these contained far fewer  
individual protoplasts than the aggregates for other  
regions.

Table (6)

The quantity of the Uvitex BOPT adsorbed by the protoplasts of tip, middle and basal segments ( $10^3$  each) after the first 4 hours of culturing.

| <u>Segments</u> | <u>Quantity of stain (<math>\mu\text{g}.\text{ml}^{-1}</math>)</u> |
|-----------------|--|
| Tip             | 2.52   |
| Middle          | 2.08   |
| Base            | 1.62   |

Table (7)

Percentage and the number of aggregated protoplasts produced from tip, middle and base segments.  $10^6$  protoplasts  $\cdot \text{ml}^{-1}$  medium were used

| <u>Segments</u> | <u>Percentage</u><br>% | <u>Number of chain</u> |
|-----------------|------------------------|------------------------|
| Tip             | 96                     | 1                      |
|                 | 4                      | 2-3                    |
| Middle          | 90                     | 8-12                   |
|                 | 10                     | 2-3                    |
| Base            | 96                     | 15-20                  |
|                 | 4                      | 2-3                    |

Aggregation is also shown in Plates (42(2,4,5), 43(1,2)). The new wall around the protoplast is not apparent but the absorbance of stain (at 350 nm) shows increase. This may be due to the less sensitivity of the microscopic technique. In addition to the aggregation, middle and basal protoplasts showed bi-polar development of hyphae, Plate (43(3,4,5,6)), while polar growth was seen during regeneration to tip protoplast, Plate (42(5,6,7,8)). The regeneration percentages of the protoplasts from different segments is shown in Figure (26). It was found that the quantity of protoplasts regenerating declined from the tip segments to the basal segments.

Usually the hyphae emerged from the protoplasts which had already formed a new wall around themselves. No development was apparent without such a new formed wall. The observation correlates with that on regenerating sporangiophores, where regeneration took place at the cut site after wall closure. The activity of the synthesis during the development of hyphae is shown in Table (8).

Hyphae developed quicker from the protoplasts of the tip segments than from those of the middle and basal segments. The slightly greater staining of the hyphae of the tip protoplasts may be attributed to the activity of these protoplasts giving more rapid wall synthesis. Cell

Table (8)

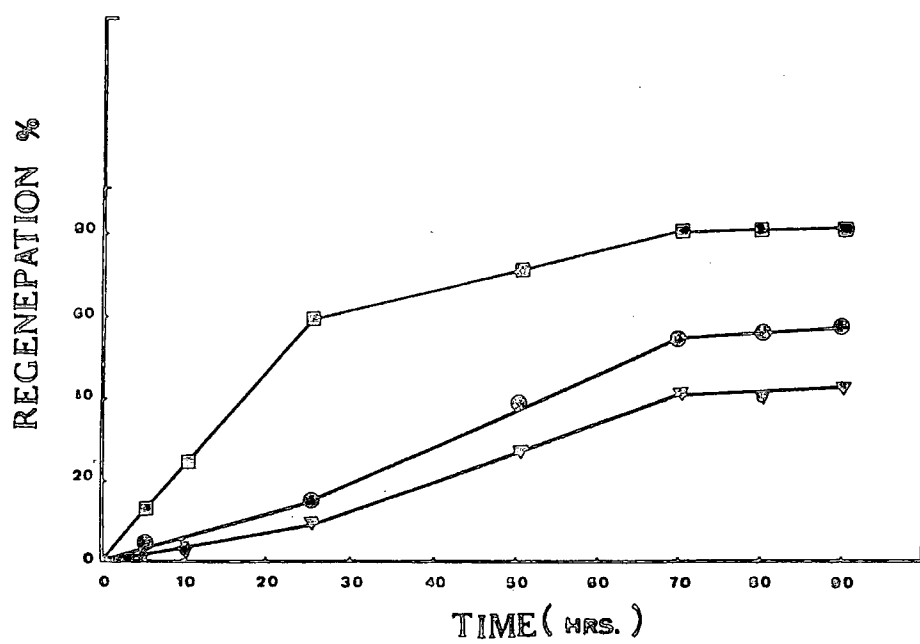
The quantity of stain Uvitex BOPT adsorbed by the hyphae developed from protoplasts of the tip, middle and basal segments at 12 hours and 24 hours.

| <u>Segments</u> | <u>Quantity of stain (<math>\mu\text{g}.\text{ml}^{-1}</math>)</u> |                 |
|-----------------|--|-----------------|
|                 | <u>12 hours</u>  | <u>24 hours</u> |
| Tip             | 10.2   | 81.6            |
| Middle          | 8.34   | 86.4            |
| Base            | 7.81   | 82.8            |

Figure (26)

Regeneration of protoplasts of different segments.  
Known numbers of protoplasts ( $10^6 \cdot \text{ml}^{-1}$ ) were cultured in a shallow layer of osmotic stabilized regeneration medium for 1-2 hours and then transferred to the solid medium. Developed protoplasts were counted and then the regeneration percentages were calculated

- Tip protoplasts
- Middle protoplasts
- ▽ Base protoplasts



regeneration of the intact tip segment of sporangiophores was also found to be achieved in a short time than that for the middle and basal segments (see Chapter I). Thus the rate of protoplasts regeneration appears to be influenced by their site or origin.

Location of the synthesis of the new wall at the tip region of the regenerated hyphae can be indicated by the presence of more stain intensity of Uvitex BOPT, Plate (39(2)). This is similar in location to the intensity of the stain at the tip segment of the intact sporangiophore.

The differences in protoplasts release and activity for different segments of sporangiophores, indicates that there could be compartmentlization and differential distribution of cellular components along the coenocytic sporangiophore. The results for this regeneration of sporangiophore protoplasts are similar to those already described for intact and segmented sporangiophores as reported in Chapter I.

The differences that were found during the regeneration of segments from the tip, middle and base of sporangiophore are in agreement with the distribution of activity and apical dominance of the original sporangiophore. Also it is important to report that the different regions were



shown to be autonomous zones for the formation of the new wall. These studies of the development of the protoplasts of the tip, middle and basal segments of the sporangiophore indicated that the morphological changes might be associated with the changes in the pattern or balance of polymer synthesis.

19. DISTRIBUTION OF NUCLEI AND WALL SYNTHESIS DURING  
REGENERATION AND DEVELOPMENT

The distribution of nuclei during protoplast regeneration and development is shown in Plates (39, 44, 45). The nuclei appeared to be important with the regeneration of the protoplast and development of hyphae. They appeared initially at the site of the hyphal emergence and increased in number during the hyphal development; what appears to be nuclear division, is shown in Plates (39(3), 44(D)), but no evidence was obtained for mitotic division as found in the sporangio-  
phore (Flanagan, 1970; Frank & Reau, 1971).

Nuclei accumulation was seen to be associated with the hyphal tip where also there was more bound stain of Uvitex BOPT. Such association can be attributed to the fact that nuclei may play an important role in wall synthesis. As shown in Chapter I, the regeneration of the sporangiophore was associated with the accumulation of nuclei.

During development of hyphae, abnormal septa were sometimes formed, Plate (44(J)). Nuclei were also shown associated with the septa location. This could explain the presence of interrelationship between nuclear division and septa formation which were found in some

fungi (Clutterbruck, 1970; King & Alexander, 1970). It has also been found that the formation of the septa reflect a certain age of the hypha and contents and also require the formation of a certain number of nuclei. Septa were also formed during culturing sporangiophore, Plate (46(3)). The septum stained intensely with Uvitex BOPT so it may represent a significant physiological state of the cytoplasm since it has been reported that by closing the septa the difference between the old and the new part of the hyphae is intensified (Fencel, 1978). It may also be associated with branch formation (Trinci, 1974, 1978a). Thus septum formation as shown in Plate (44(J)), may be attributed to the branch formation beside the septum. Alternatively such observations may be an abnormal feature and may be produced by environmental change during the manipulation or may be to the age of the sporangiophores, but it can be concluded that abnormal conditions acting on nuclei or cytoplasm may also produce abnormal differentiation and morphogenesis of the organism.

20. QUANTITATIVE CHANGES IN NUCLEIC ACID AND PROTEINS  
DURING REGENERATION AND DEVELOPMENT OF THE PROTOPLASTS

During the regeneration and development of the protoplasts isolated from tip, middle and basal segments of equal lengths, 5 mm each, DNA, RNA and protein were extracted and estimated quantitatively (see materials and methods).

As shown in Figure (27) the RNA, protein and DNA of the protoplast of the tip segments reached maximum recovery in 8-12 hours, while this process occurred between 16-18 and 18-20 hours for the middle and basal segments respectively. RNA and protein contents were increased before DNA. This is similar to the situation found during the germination of Phycomyces spores (Van Assch & Carlier, 1973 and see review by Van Etten et al., 1974; Bramble et al., 1978).

The maximal recovery was seen to correlate with regeneration and development of the hyphae. So the activity of the macromolecules of the protoplast appear to be important in the initiation of regeneration. Similar observations were made during the manipulation of intact segments of sporangiophores. The initial concentration of the macromolecule of the intact segments was more than that of the corresponding protoplasts. This may be due to the effect of the isolation of the

Figure (27)

Concentration of macromolecules extracted from  
protoplasts of different segments of stage I  
sporangiophores during regeneration

1. Protoplasts of tip segments

☐ RNA

☒ protein

☐ DNA

2. Protoplasts of middle segments

☐ RNA

☒ protein

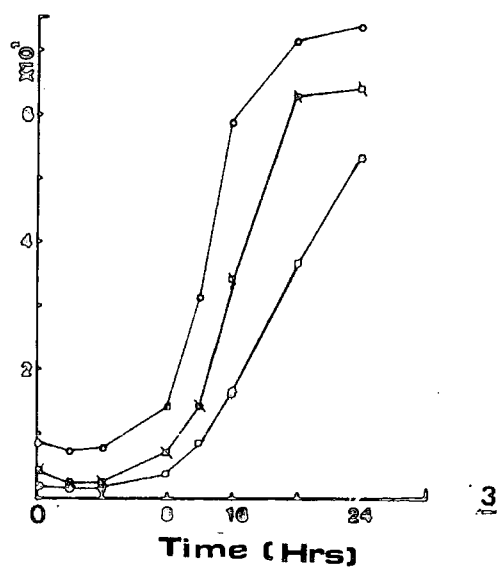
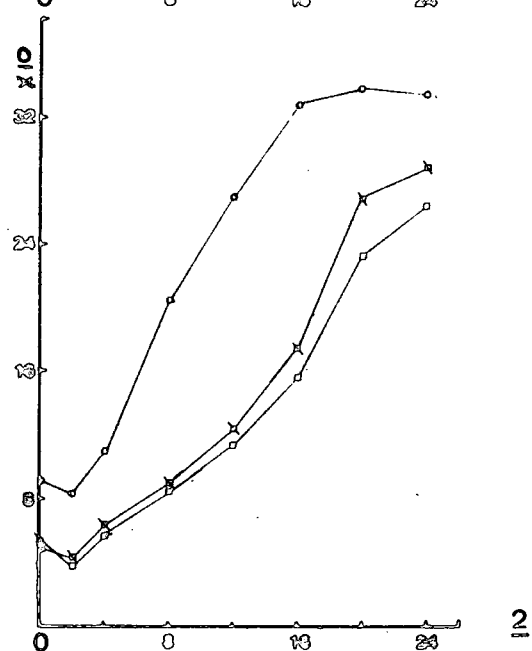
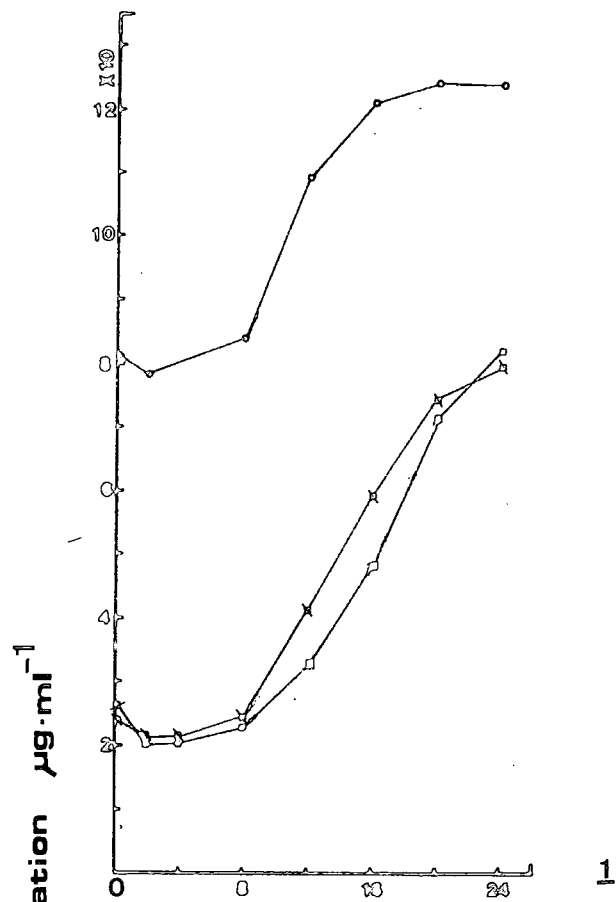
☐ DNA

3. Protoplasts of basal segments

☐ RNA

☒ protein

☐ DNA



protoplast in decreasing the total concentration of macromolecules.

21. THE EFFECT OF NUCLEIC ACID AND PROTEIN SYNTHESIS INHIBITORS

The essential requirement for DNA, RNA and protein synthesis during regeneration and development of protoplasts was tested by incubating the protoplasts in  $25 \mu\text{g}.\text{ml}^{-1}$  of each of ethidium bromide, colchicine, actinomycin D, rifampicin, 6-methylpurine, MDMP and in  $20 \mu\text{g}.\text{ml}^{-1}$  cycloheximide. At the first 2-7 hours the regeneration was not inhibited by the presence of the above inhibitors. The development of the hyphae after this time was blocked by both cycloheximide and 6-methylpurine. Thus, both RNA and protein synthesis in development appeared to be required in hyphal development. In contrast the development of hyphae continued when the protoplasts were cultured in the other inhibitors.

RNA and protein were extracted and estimated as previously (see materials and methods of Chapter I) and at 24 hours from the initial culturing in the inhibitors. The concentrations of these macromolecules are shown in Table (9). The concentrations of DNA, RNA and protein of protoplasts treated with inhibitors were less than those of the control. However, although only cycloheximide and 6-methylpurine completely inhibited the growth of regenerated hyphae, they did not have the greatest effect on the RNA and protein level. This is in agreement with the results reported in Chapter 1.



Table (9)

Total concentrations of nucleic acids and protein ( $\mu\text{g}.\text{ml}^{-1}$ ) after 24 hours of culturing protoplasts in regeneration media containing the following inhibitors. Weights of the sporangiophores were taken into account in the calculations.

| <u>Inhibitors</u> | <u>Final Stage</u> | <u>Concentrations (<math>\mu\text{g}.\text{ml}^{-1}</math>)</u> |            |                |
|-------------------|--------------------|---|------------|----------------|
|                   |                    | <u>DNA</u>  | <u>RNA</u> | <u>Protein</u> |
| Cycloheximide     | I                  | 118   | 272        | 320            |
| 6 Methylpurine    | I                  | 113   | 262        | 151            |
| MDMP              | IV                 | 130   | 115        | 286            |
| Actinomycin D     | IV                 | 123   | 250        | 275            |
| Rifampicin        | IV                 | 135   | 221        | 325            |
| Ethidium bromide  | IV                 | 98  | 265        | 308            |
| Colchicine        | IV                 | 100   | 254        | 359            |
| Control           | IV                 | 125   | 267        | 362            |

The inhibition of complete regeneration of the protoplasts of Schizophyllum commune by cycloheximide was also found by Vries & Wessels (1975) who attributed the initial regeneration seen in the presence of the inhibitor to the pre-existing chitin synthase since protein synthesis was almost totally inhibited. Inhibition of the synthesis of chitin precursors by cycloheximide and 5-fluorouracil was also correlated to the inhibition of the cell wall formation during spore germination of Phycomyces, see Van Laere et al., (1976, 1977). Thus it may be concluded that the initial regeneration of freshly isolated protoplasts is not dependent on de novo synthesized protein.

Synthesis of some protein in the presence of cycloheximide has been found to be associated with the loss of control of polarity and inhibition of the lytic enzyme in Aspergillus nidulans (Wang & Bartnicki-Garcia, 1966; Katz & Rosenberger, 1971); similar observations also can be implicated for Phycomyces treated with cycloheximide. Thus inhibition of the synthesis of specific proteins which are required for growth may have been taking place under the influence of the applied inhibitors. However, it must be born in mind that the activity of the inhibitors may not be specific. The lack of an effect of an inhibitor on sporangiophore development being possibly

due to a lack of penetration through the wall was suggested in Chapter I, but work with naked protoplasts gave similar results, therefore a limitation of penetration may not be the reason of the lack of effect.

22. INFLUENCE OF MEDIA ON SPORANGIOPHORE AND DERIVED PROTOPLASTS REGENERATION

Plucked stage I sporangiophores were cultured by immersing the basal ends in liquid media, which were either carbon rich or nitrogen rich. After regeneration for 10-12 hours sporangiophores were fixed in acetic acid-alcohol and stained with acridine orange and then examined with a fluorescent microscope, see Plate (47). It was found that the basal end of the sporangiophore cultivated in the nitrogen-rich medium regenerated to give more hyphae compared with those with the cultivated end in the carbon-rich medium. Also, the diameters of the hyphae which regenerated in the carbon-rich medium were found to be greater than those which regenerated in the nitrogen-rich medium. Also, less branching was found during the development of hyphae from the basal end of sporangiophores which were cultured in the carbon-rich medium.

In the nitrogen rich medium, the hyphae that developed appeared to be produced from sites which had the form of protoplasts. Each hypha appeared to develop separately during the initial regeneration. In contrast, in those grown in the carbon-rich medium the whole bulk of the cytoplasm at the basal end developed an unbranched regeneration which appeared to be formed from one large protoplast-like structure. Similarly protoplasts

cultivated in the nitrogen-rich medium also showed greater regeneration and more developed hyphae compared with those cultured in the carbon-rich medium, Plates (46(1,2,4), 48(3,4), 49(3,4)). Developed hyphae in the nitrogen-rich medium also formed more branches, Plate (4(1,2)) compared with those which were cultured in the carbon-rich medium, Plate (49(1,2)) and Plate (46(4)). Thus the protoplasts and the sporangiophore behaved in a similar fashion in response to media changes.

23. THE EFFECT OF HOMOGENIZATION ON THE REGENERATION OF PROTOPLASTS

The effect of homogenization with destruction of the normal arrangements of cytoplasmic organelles was tested, the context being that it can be compared with the effect of cutting in activating branching of the sporangiophore, as shown in Chapter 1. It was suggested that the cutting may have resulted in the release of some substance(s) which controlled the regeneration and thus branching.

Isolated protoplasts from stage I sporangiophore were homogenized in a pestle and mortar thus breaking the membrane and disrupting the distribution of organelles. Treated protoplast were cultured in both solid and liquid minimal medium. Plate (50(1,2)) shows the regeneration of treated protoplasts which appeared different from regeneration of intact protoplasts (see above). The regeneration appeared to be initiated from granular structure since no typical unit membrane profiles appeared. More than one developed hypha emerged from each structure. This could be attributed to the formation of chitin microfibrils, which could be intimately associated with granules. An alternative explanation was reported by Ruiz-Herrera et al., (1975) in terms of microfibril assembly by granules of chitin synthase in Mucor rouxii.

The granular system appeared to emerge as a result of the breaking of the integrity of the cytoplasmic membrane. This may be associated with the release of the bound or latent cell wall synthesis enzyme which was controlled by the cytoplasmic membrane during the normal growth of the protoplasts. The effect of the cut or injury of the segments in producing regeneration and branching of sporangiophore may be explained by the fact that the cutting may have activated or released the membrane-bound enzyme and thus produced a random growth.

A particulate chitin synthase of Phycomyces has been isolated (Thomson & Fischer, 1976) in an inactive or zymogen state. Also, Thomson & Fischer (1976) have found that incubating the enzyme with trypsin or crude preparations of a neutral protease (an endopeptidase) from Phycomyces led to a 10 fold activation of chitin synthase. The observations of regeneration from a granular structure could represent the particulate chitin synthase which might be activated during the homogenization.

Regeneration from a granular like structure was also found without homogenization. It was achieved by the cytoplasmic components, which were present inside the sporangiophore which had been cultured in the nitrogen-rich medium, Plate (50(3)), and the structure of

regeneration found here resembled that which formed after homogenization. Autolysis of the cytoplasmic organelles, inside the sporangiophore in which hyphae were regenerated from a granular form may have played a part. During autolysis substances may have been liberated into the cytoplasm and may have activated the particulate chitin synthase of Phycomyces (see Thomson & Fischer, 1976; Fischer & Thomson, 1979), since the isolated sporangiophore was in a condition of starvation which showed induction of autolysis (see Fenc1, 1978).

Increased branching at the tip region may be attributed to the presence of more concentrated membrane-bound enzyme(s) (see Jan, 1974). Cutting may have caused a release of some substances which may have activated chitin synthase (Fischer & Thomson, 1979). This finding supported the hypothesis of Ruiz-Herrera et al., (1975) that the granular structure may elaborate the cell wall microfibril. In this case the presence of less granular cytoplasm in the basal and middle sections of sporangiophores may be attributed to less activity of wall synthesis while the granular cytoplasm was more concentrated at the tip of the sporangiophore.



Plate (34)

Regeneration of protoplasts on solid medium

1. At zero hour

2. At 4 hrs, showing formation of hyphae aggregates on the surface of protoplasts

magnification X2600

3. At 6 hrs, showing an increase in diameter during regeneration

magnification X2600

4. At 8 hrs, showing the irregular and peripheral regeneration

magnification X2800

6. At 10 hrs, showing the peripheral regeneration and vesicle like structure formation

magnification X2800

8. At 12 hrs

magnification X2800

BA: Bulging Area

Mic: Granular material presumed to be aggregates of microfibrils

P: Protoplast

OP: Original protoplast

HT: Hyphal Tip

V: Vesicle like structure

Pr: Protrusion, this may be attributed to the formation of polymicrofibrils

RH: Regenerated Hypha

Rp: Regenerated protoplast

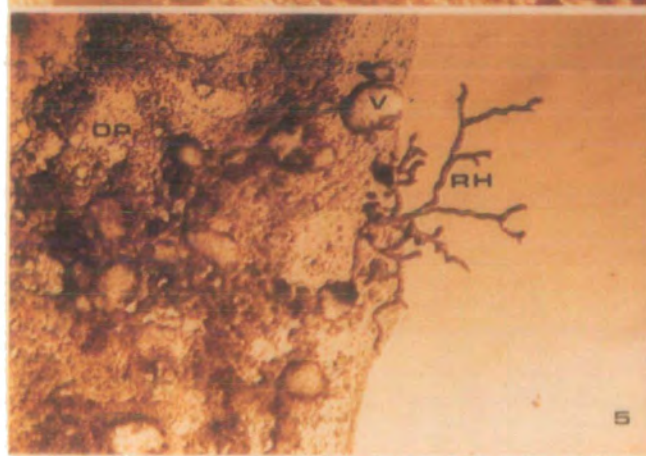
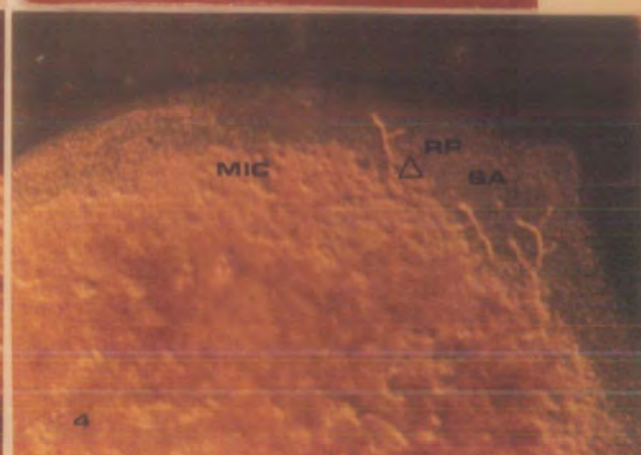
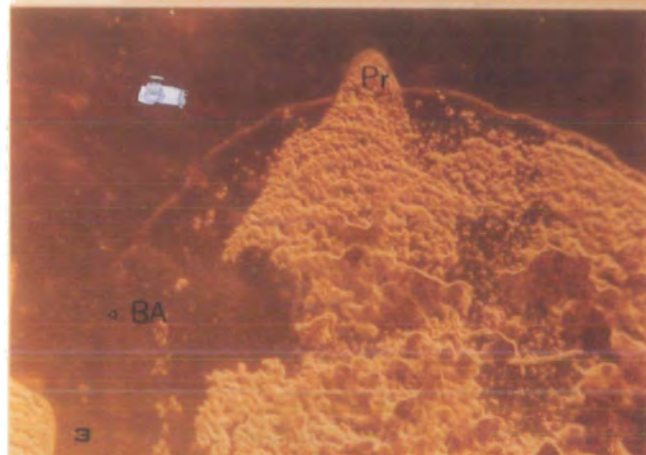
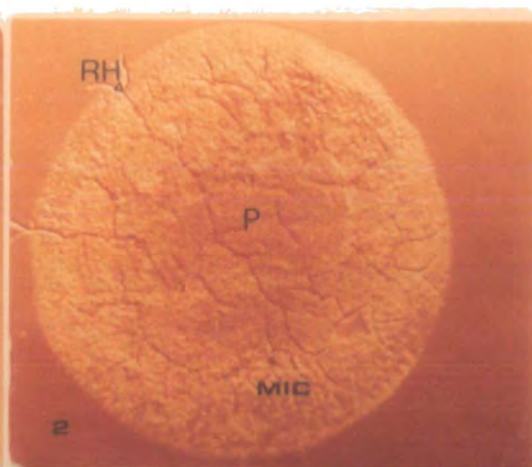
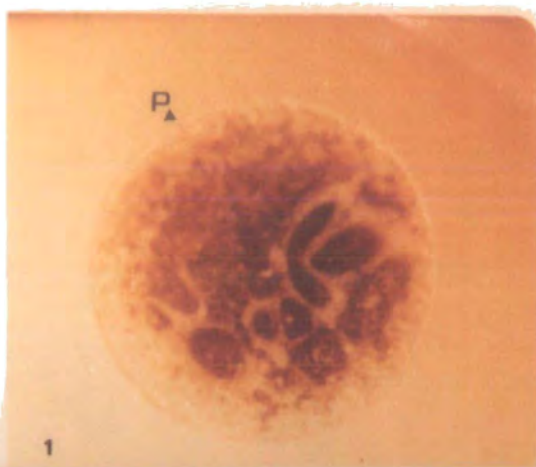


Plate (35)

Regeneration of protoplasts on solid medium

- 1 & 2. Showing the formation of the hyphae, covering the surface of the protoplast. Hyphae can also be seen, emerging from the peripheral region

1 - magnification X2600

2 - magnification X3600

- 3,4 Showing the development of hyphae from the regenerated protoplasts and also the formation of foot cells, at 24 hours after culturing

magnification X800

- 5 Showing the development of sporangiophores from foot cells

magnification X800

Fc: Foot cell

HA: Hyphal aggregation

OP: Original Protoplast

P: Protoplast

RH: Regenerated Hyphae

RP: Regenerated Protoplasts



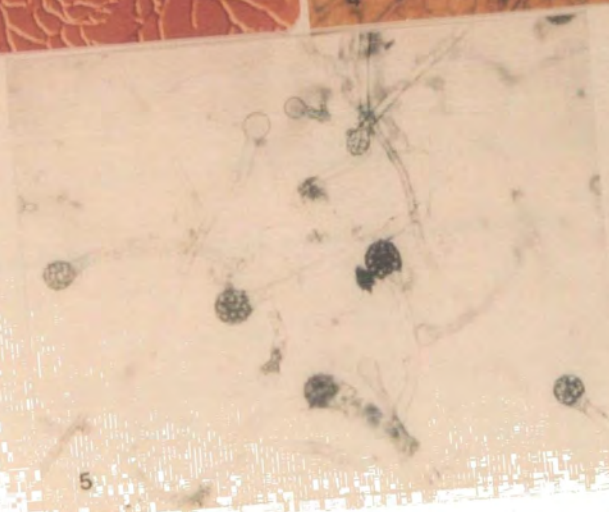
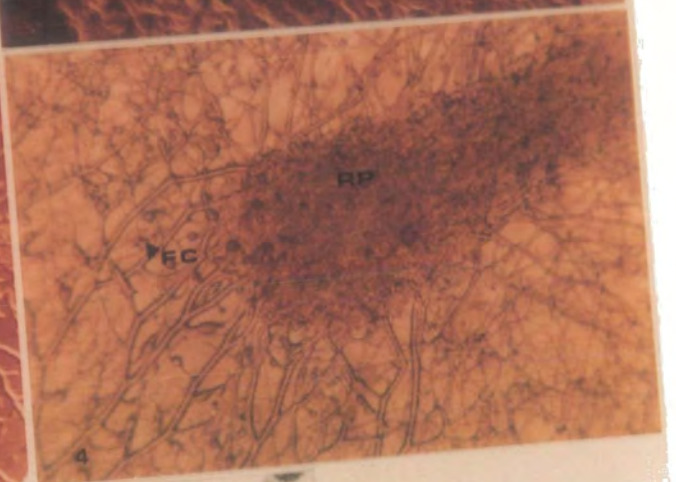
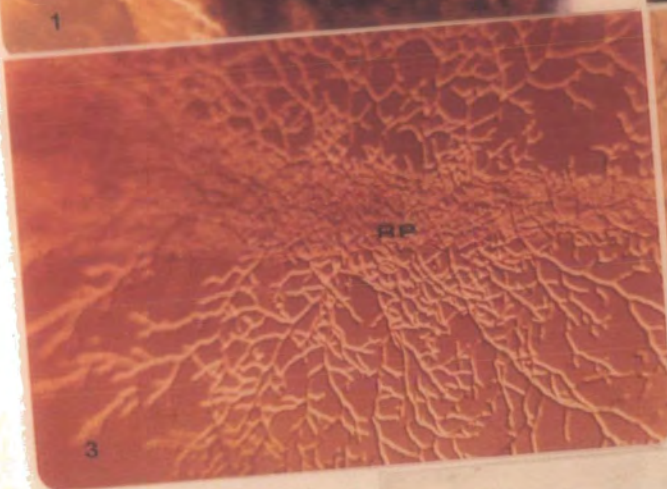
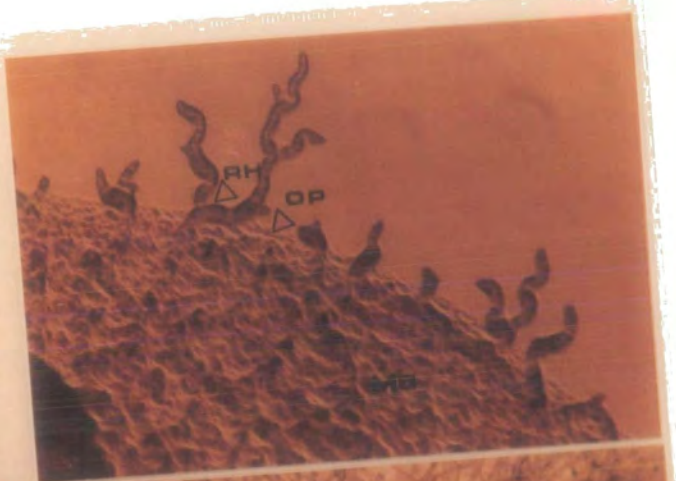
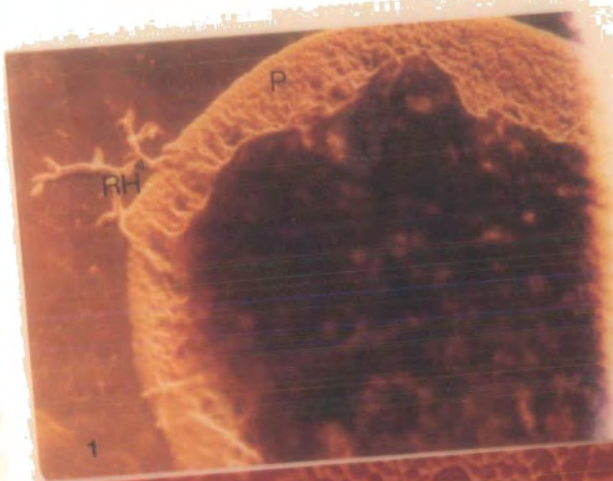


Plate (36)

Development of hypha from protoplasts cultured on the solid medium

1. Peripheral or marginal region. Protoplasts were stained with 0.1% solution of pyronin Y for 5 minutes

magnification X52

2. Central region. Protoplasts were stained as in 1.

magnification X400

3. Stained with toluidine blue and showing clumping

magnification X600

4. Stained with acridine orange and showing nuclei

magnification X2000

HT: Hyphal Tip

N: Nuclei

RH: Regenerated Hypha



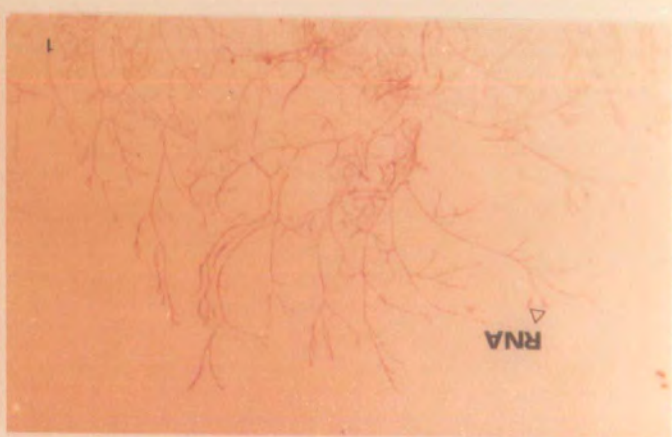
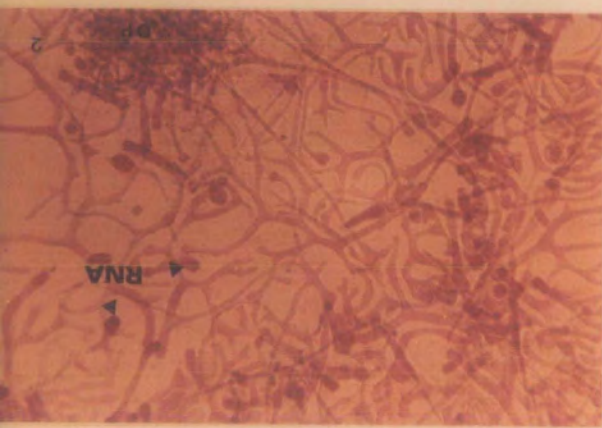
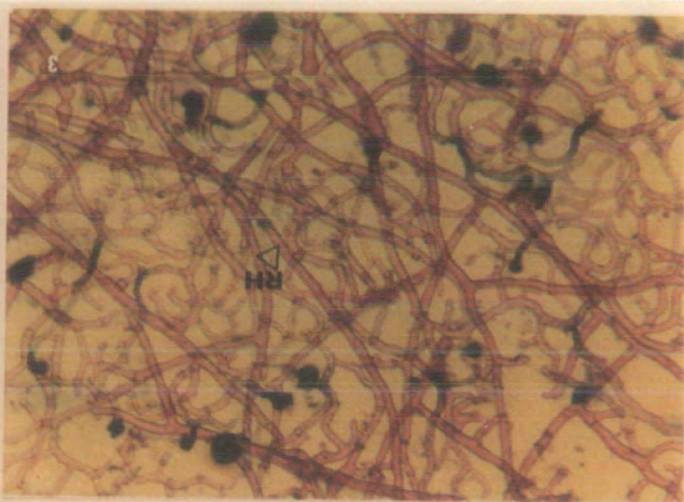
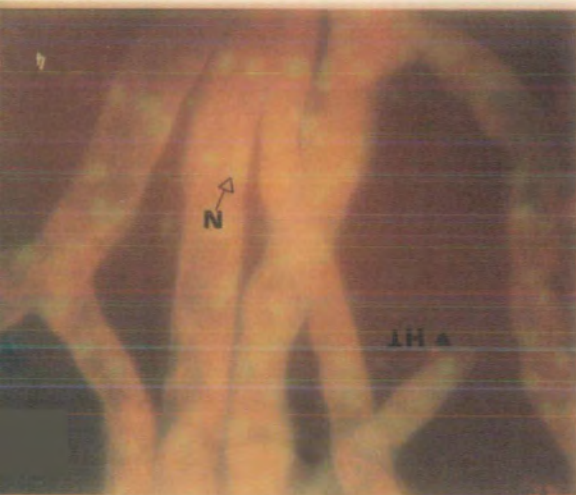


Plate (37)

Development of hyphae from protoplasts cultured on the solid medium

1. Peripheral region showing tips leading to hyphae

magnification X320

2. Middle and central mature region.  
Fixed with acetic acid-alcohol and stained with Giemsa, showing foot cell formation

magnification X400

3. Sporangiphore developed from the globular body (foot cell)

magnification X52

Fc: Foot cell

H: Hyphae



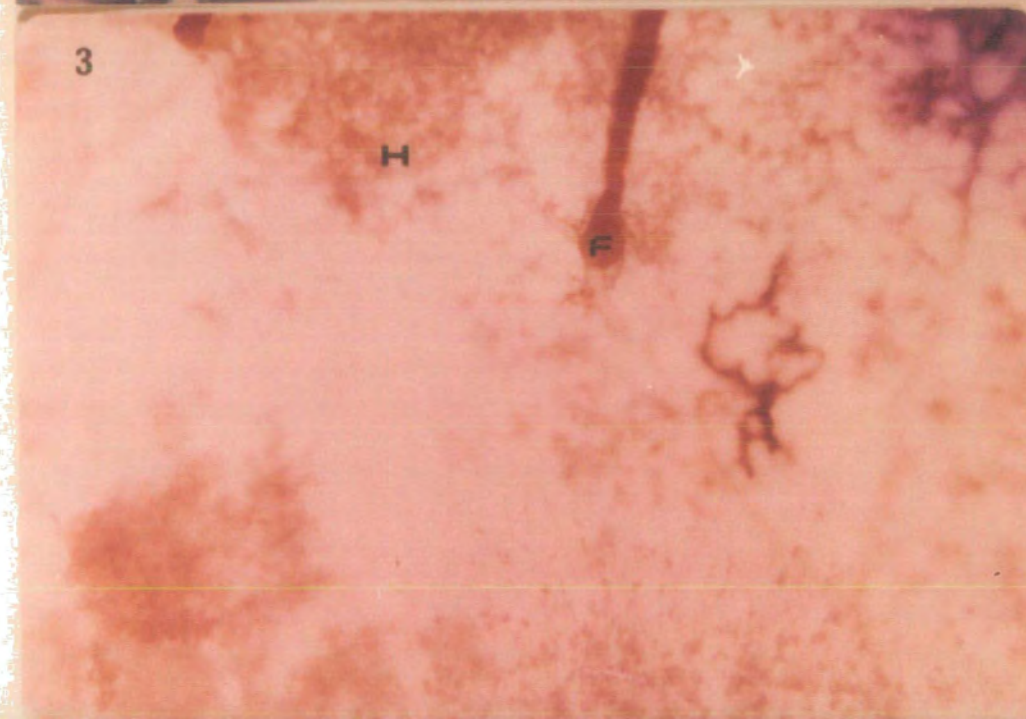
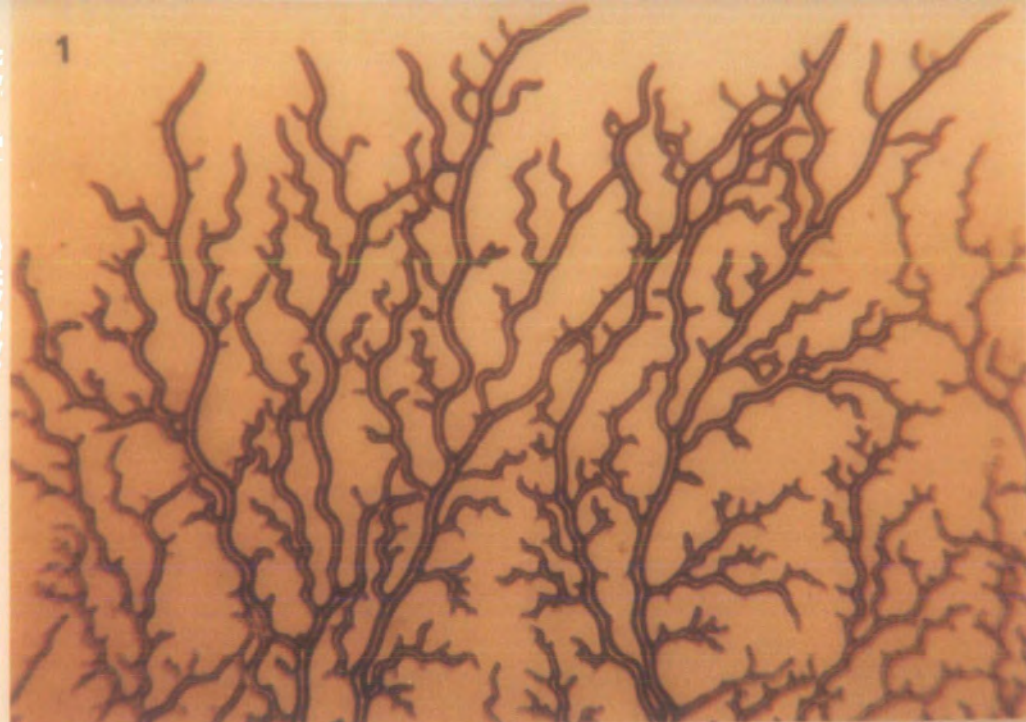




Plate (38)

Regeneration and development of protoplasts. Preparations in 1-4 were fixed in acetic acid-alcohol and stained with acridine orange

In liquid medium

1. Bud formation

magnification X3125

2. Aggregation

magnification X3125

In semi solid medium

3. Globular and aberrant hypha formation

magnification X1225

4. Formation of chain like cells

magnification X2700

5. Bursting of regenerated protoplasts after replacing in water

magnification X1225

AH: Aberrant Hypha

B: Budding

PA: Protoplast Aggregation

P: Protoplast

N: Nuclei

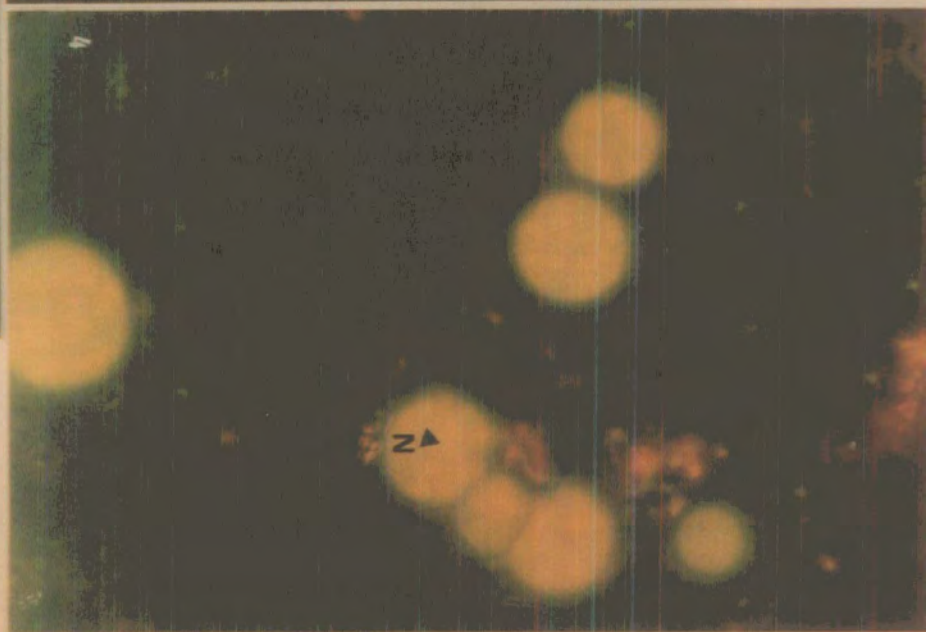
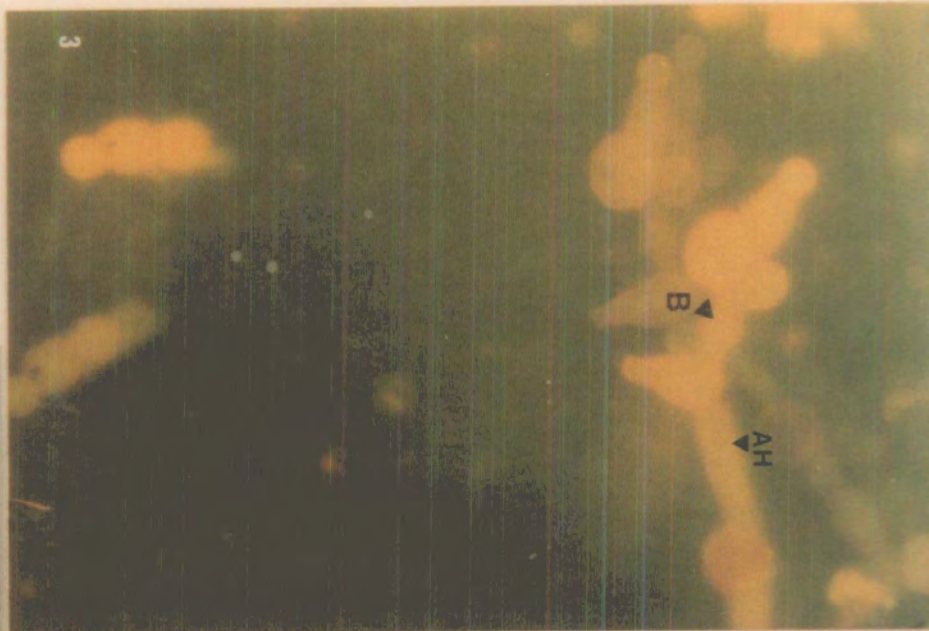


Plate (39)

Protoplast regeneration and development during culturing, submerged in liquid stabilized minimal medium. Developed protoplasts were fixed in acetic acid-alcohol and stained with acridine orange

1. At 2 hours
2. At 6 hours, showing polar growth and development of hyphae
3. At 12 hours
4. At 16 hours, and showing constriction of hyphae
5. At 24 hours, notice greater intensity of stain for DNA during formation of septa
6. At 28 hours, showing bud chain formation, or yeast like cells

1 - 2 magnification X1250

3 - 4 magnification X2250

5 - 6 magnification X1250

C: Constriction

N: Nuclei

OP: Original Protoplast

P: Protoplast

Pr: Protoplast regenerating to hyphae

RH: Regenerated Hyphae

YC: Yeast like Cell

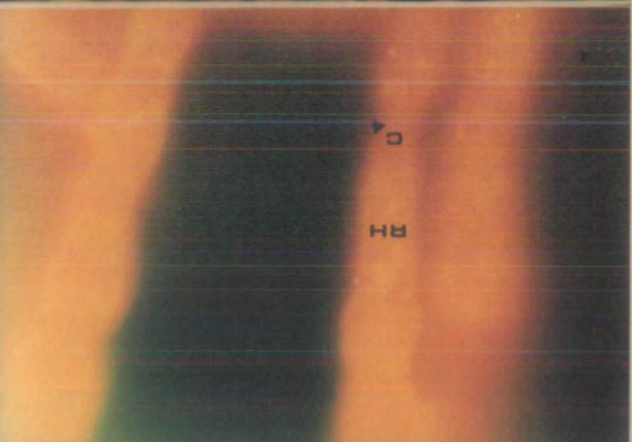
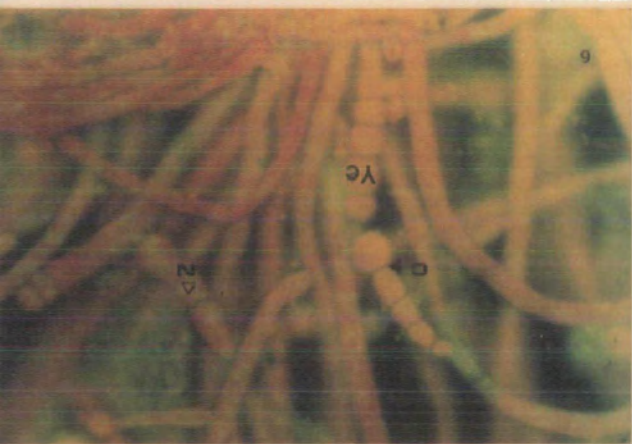
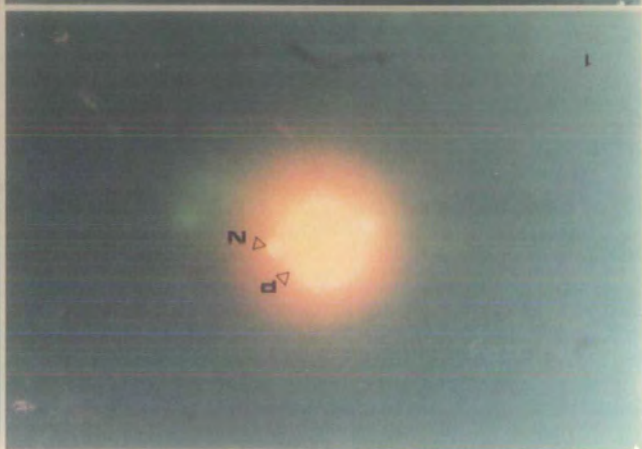
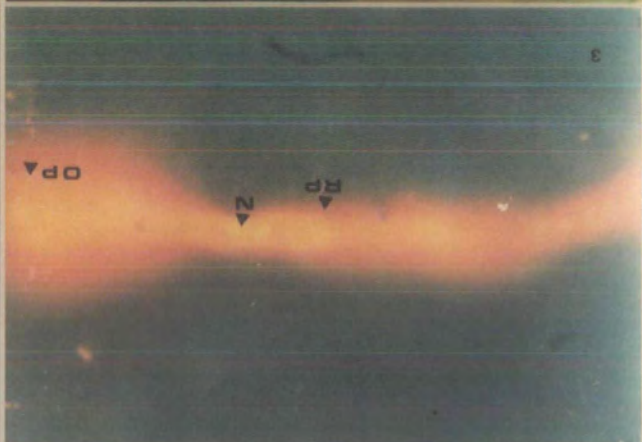
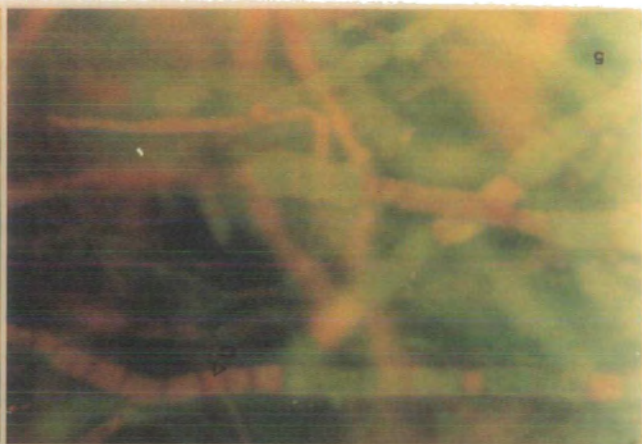


Plate (40)

Distribution of wall binding stain (Uvitex BOPT) and nuclei during development of protoplasts

1. At 8 hours, stained by acridine orange and Uvitex BOPT in combination

magnification X6125

2. At 12 hours, stained by acridine orange and Uvitex BOPT in combination

magnification X6125

3. At 24 hours, stained with acridine orange. Red background indicates RNA

magnification X7000

N: Nuclei

OP: Original protoplast

P: Protoplast

V: Vacuole



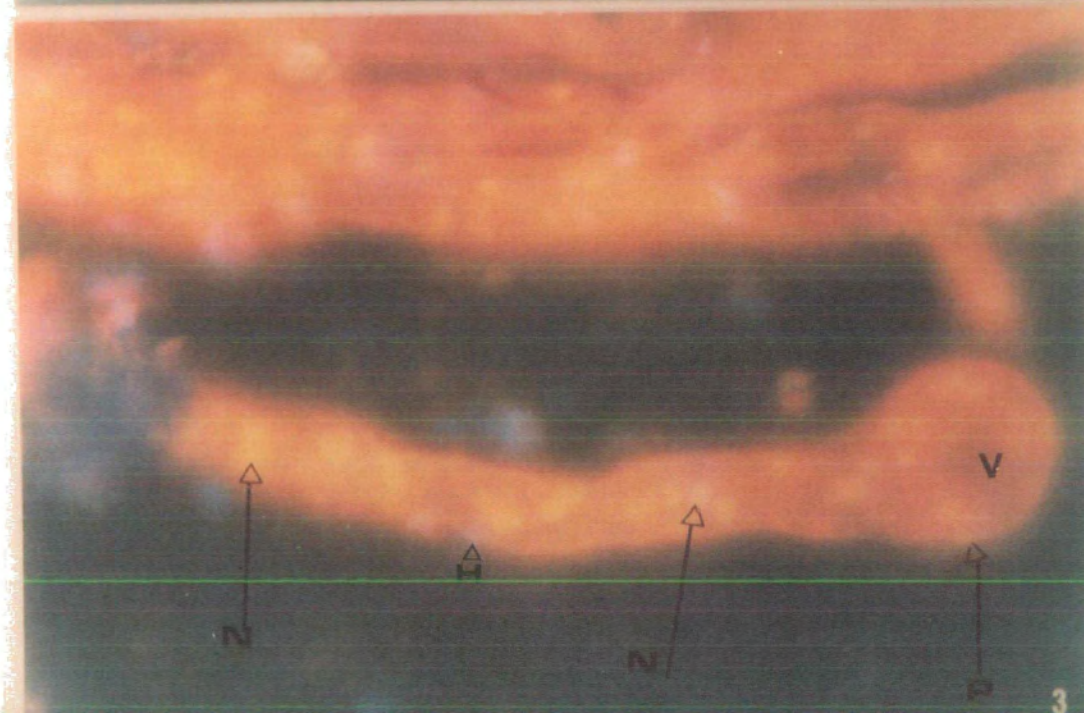
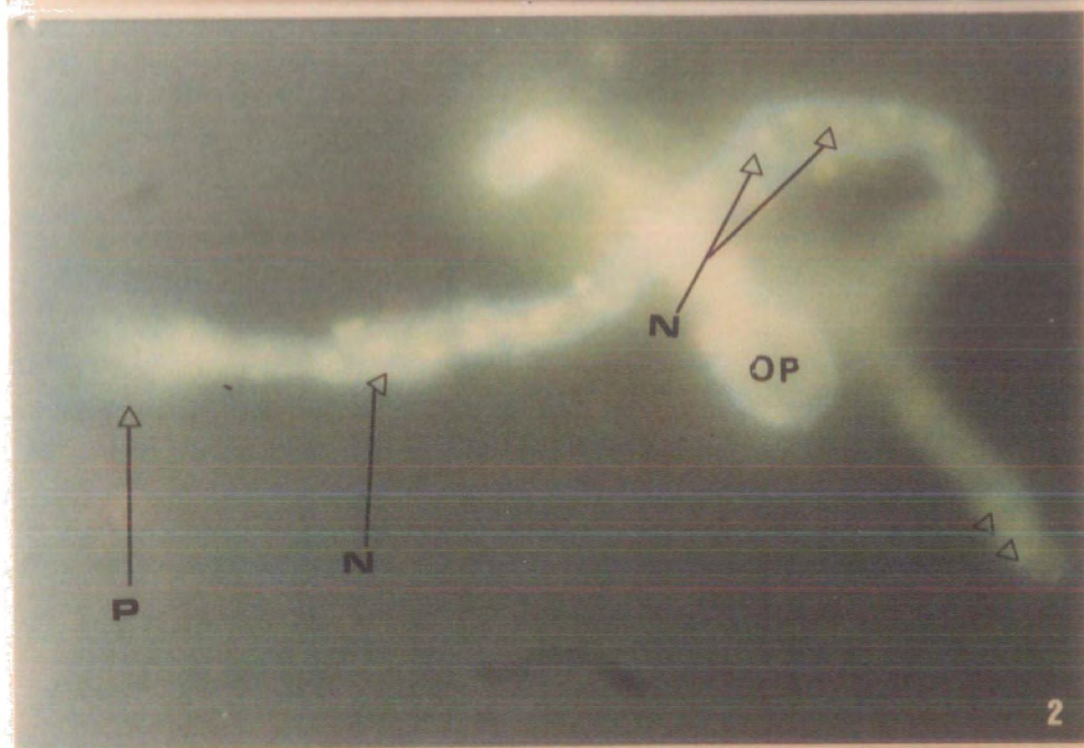
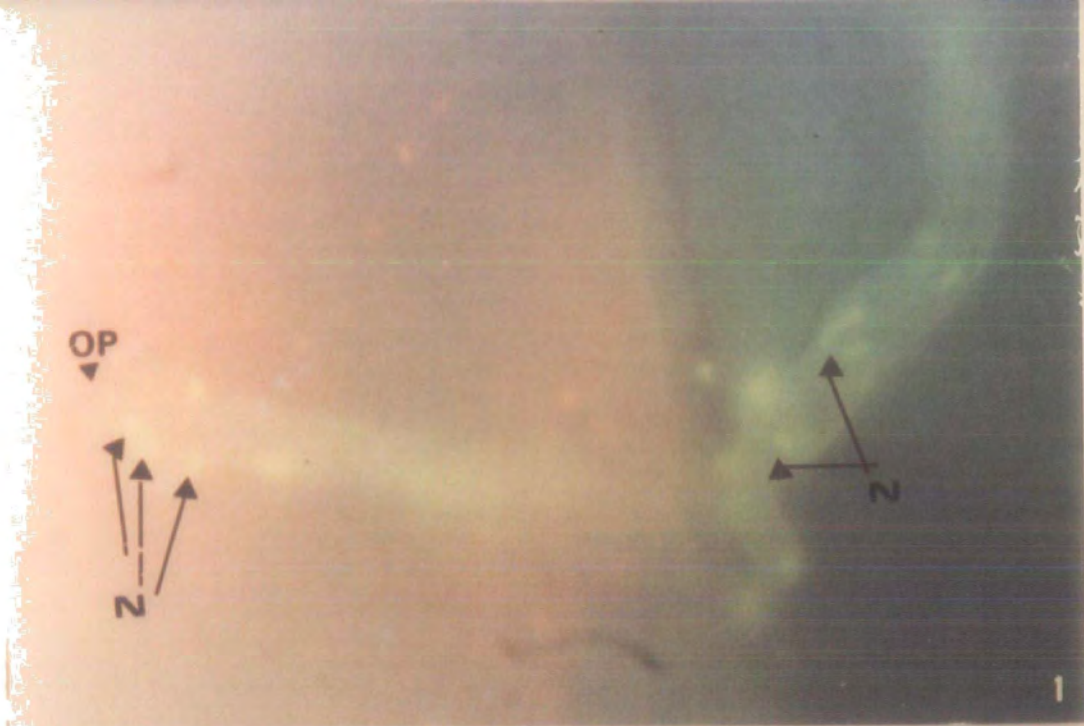


Plate (41)

Aggregation and development of protoplasts during  
culturing in liquid medium for 6 hours

1 - magnification X320

2 - magnification X1280

RI: Regeneration Initiation

Spph: Sporangiphore

TC: Tubular Cell

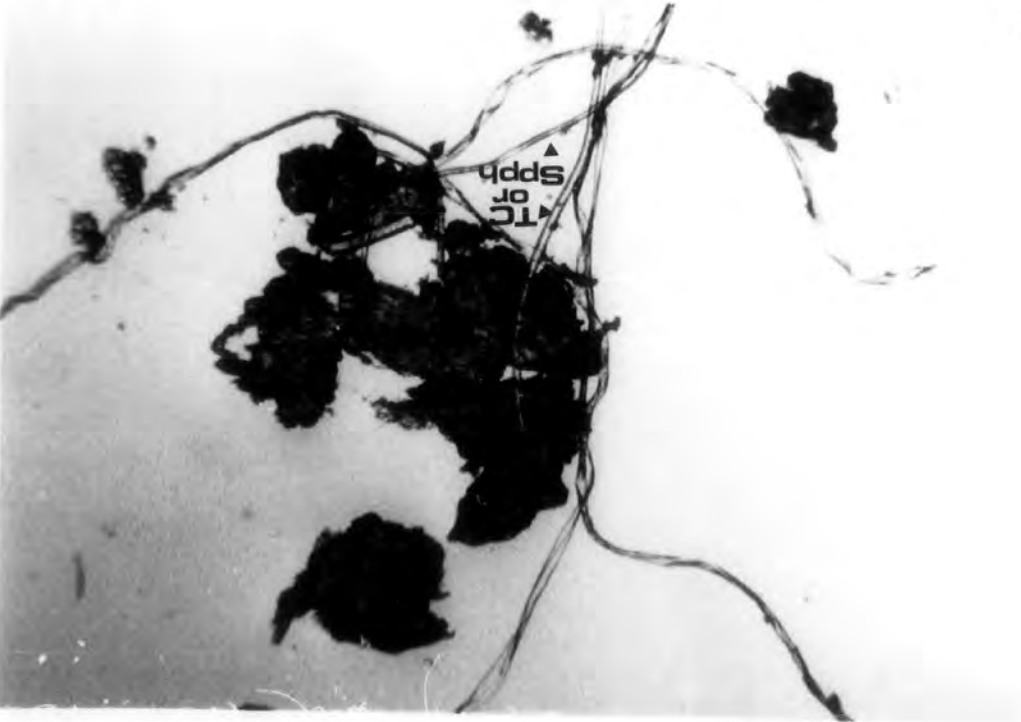
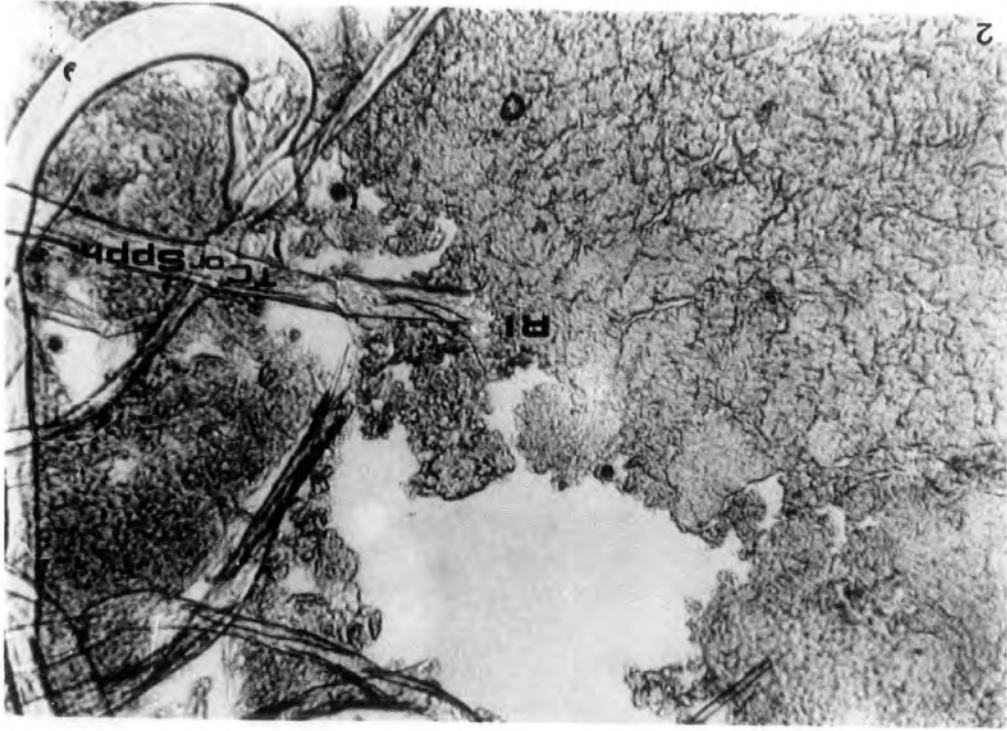




Plate (42)

Regeneration and development of protoplasts in stabilized liquid medium

1. Linear aggregation of protoplasts and showing formation of tubular cell

magnification X400

- 2,3,4 Formation of yeast like cells and aberrant hyphae

magnification X320

- 5,6 Subsequent development of hypha from the protoplast, released from tip region

- a - 0 hour
- b - 2 hours
- c - 4 hours
- d - 6 hours

5 - magnification X1000

6 - magnification X320

- 7,8 Development of one or two hyphae from the protoplast released from the tip region

magnification X1600

AH: Aberrant Hypha

AP: Aggregation of Protoplast

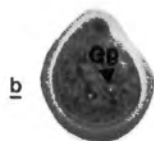
CB: Circular Body

RW: Regenerated Wall

YC: Yeast like Cell

OP: Original Protoplast

GB: Globular Body like structure



5

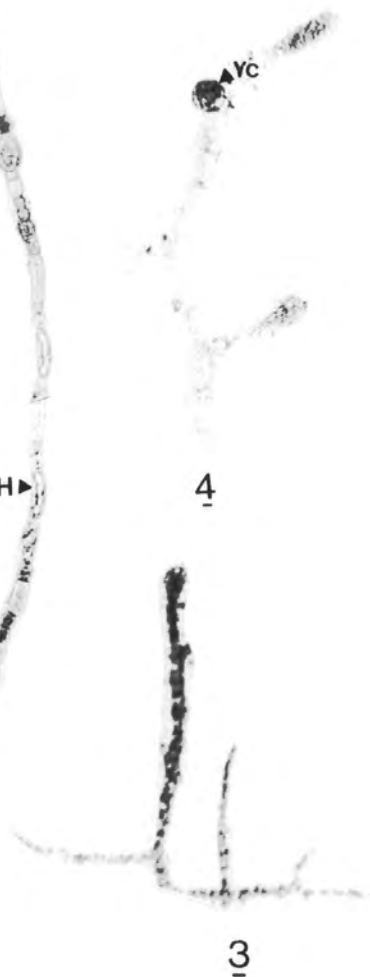


Plate (43)

Regeneration of protoplasts, cultured in liquid medium

1 - At 2 hours

showing aggregation of protoplasts

2 - 4 hours

3 - 6 hours

4 - 8 hours

showing development of hyphae from  
protoplast

5 - 10 hours

showing bi-polar growth

6 - 12 hours

magnification X1280

AP: Aggregated Protoplasts

RH: Regenerated Hypha

RP: Regenerated Protoplasts

BiR: Bi Regeneration

OP: Original Protoplast

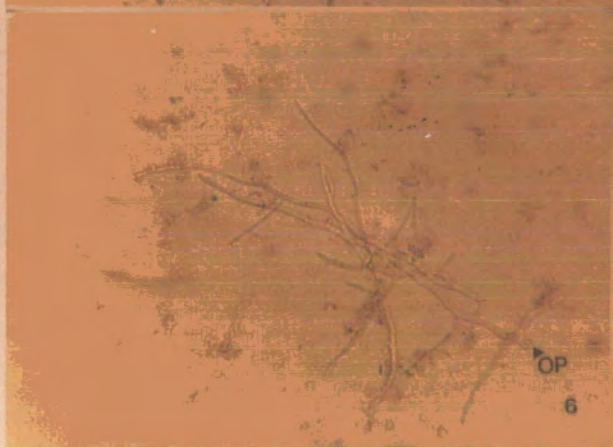
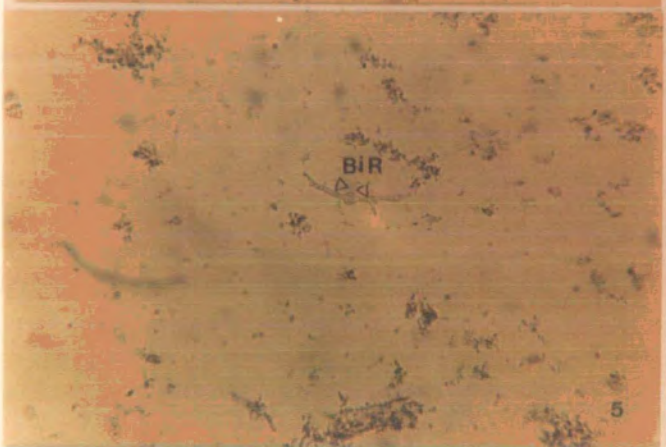


Plate (44)

Distribution of nuclei after regeneration of protoplasts  
in liquid medium, and stained with Feulgen stain

A - E - 8 hours

magnification X1600

F - 10 hours

magnification X1800

G - 12 hours

magnification X1600

J - L - 20 hours

magnification X1800

A: Apex

N: Nuclei

GB: Globular Body

Se: Septum

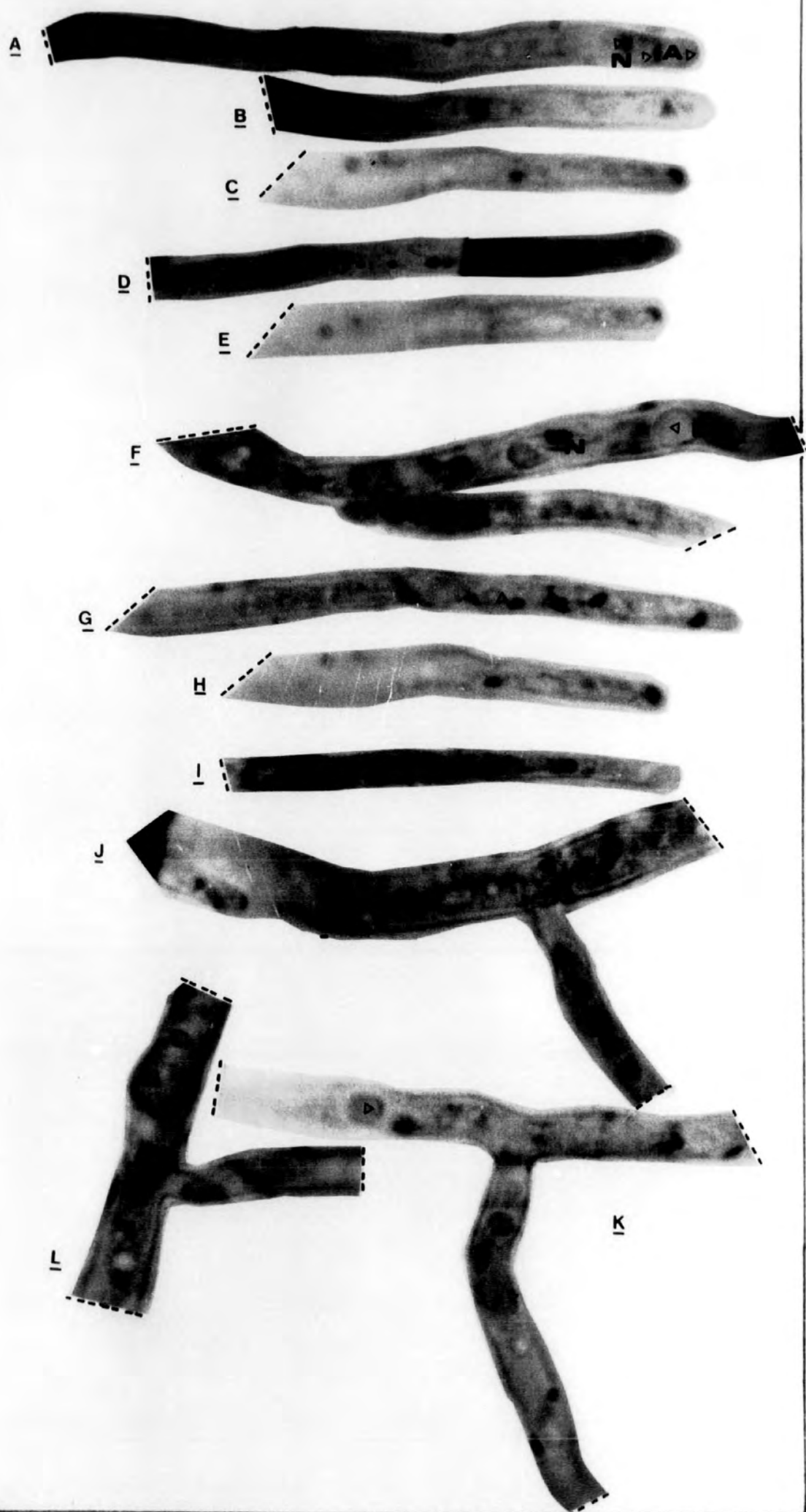


Plate (45)

Distribution of nuclei along developed hyphae from protoplasts. Protoplasts were cultured on cover slip; fixed in situ by acetic acid-alcohol and stained with acridine orange. Greater accumulation of nuclei can be seen at the tip region of the hypha

magnification X3000

H: Hypha

N: Nuclei



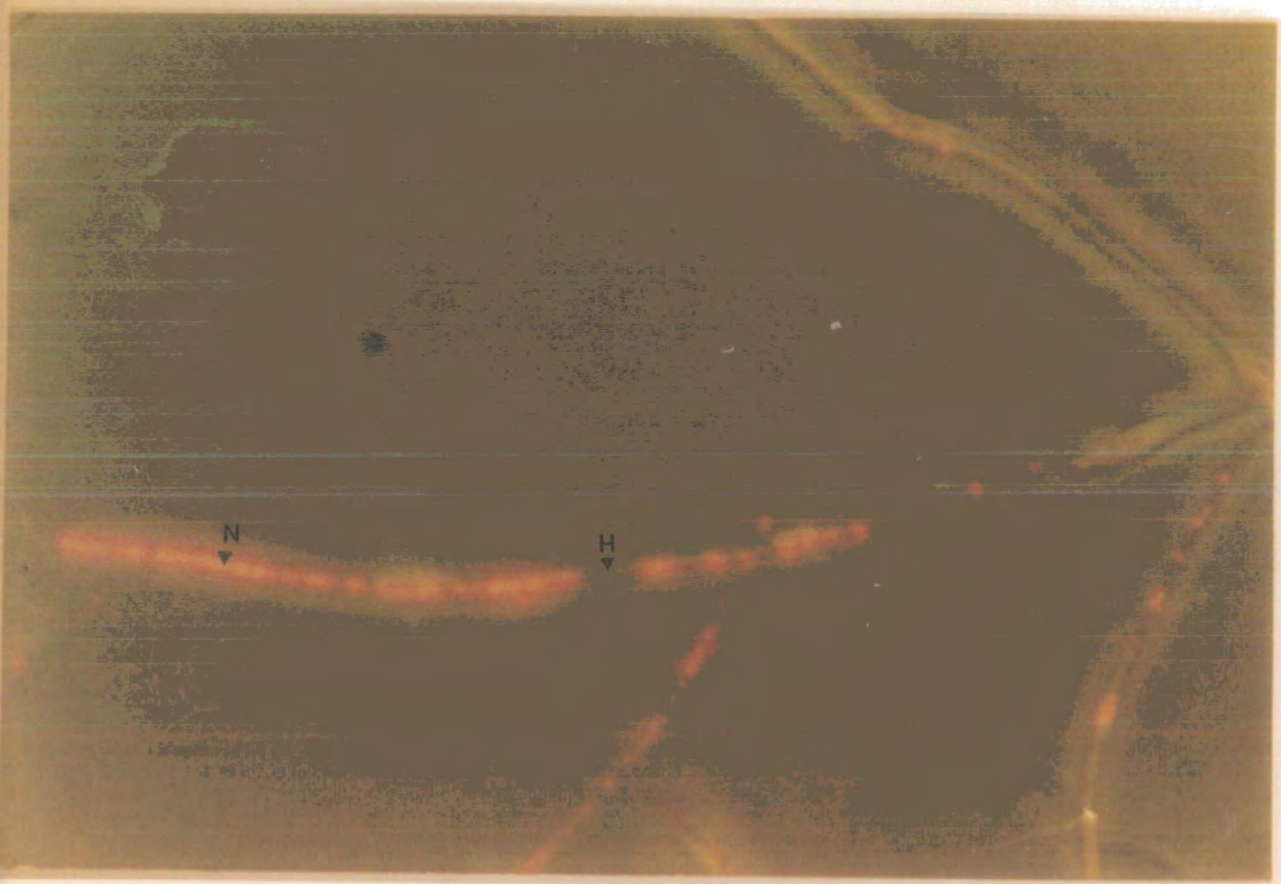




Plate (46)

- 1,2    Regeneration of protoplasts in nitrogen-rich medium. Protoplasts stained with Uvitex BOPT and showing a greater yield of developed hyphae

1 - at 12 hours

2 - at 24 hours

magnification X1000

- 3a    Regeneration of protoplast in carbon-rich medium at 24 hours

magnification X3125

- b    Formation of septum across the sporangiophore

magnification X1125

- 4    Regeneration of protoplast in carbon-rich medium

magnification X1125

GT: Growth Tip

P: Protoplast

Se: Septum

Spph: Sporangiophore

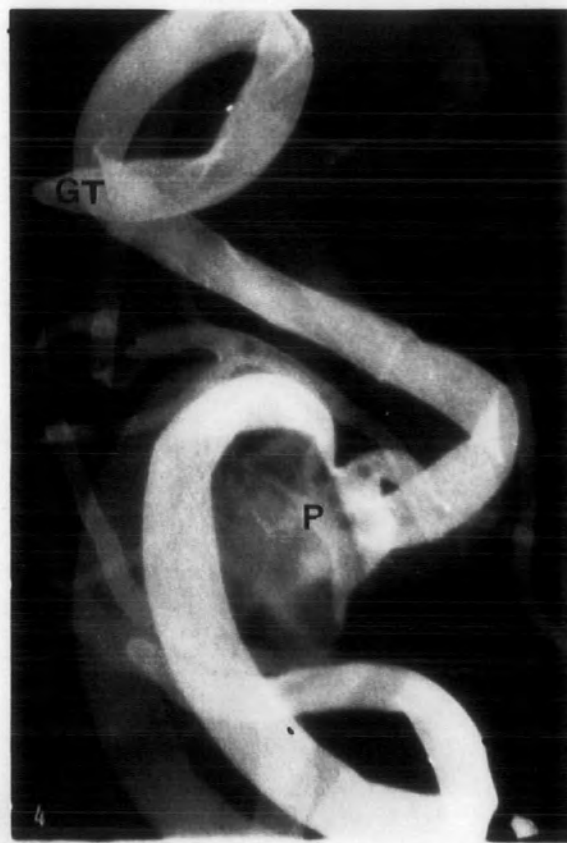
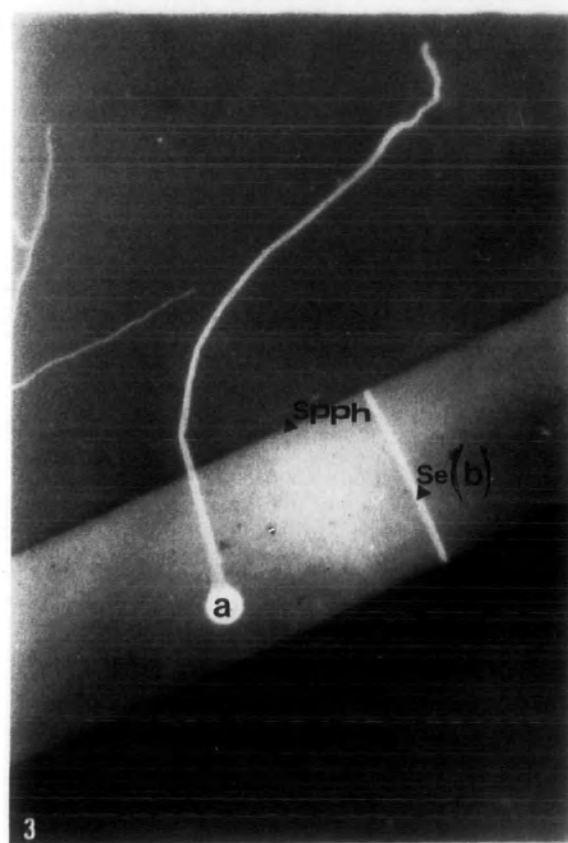
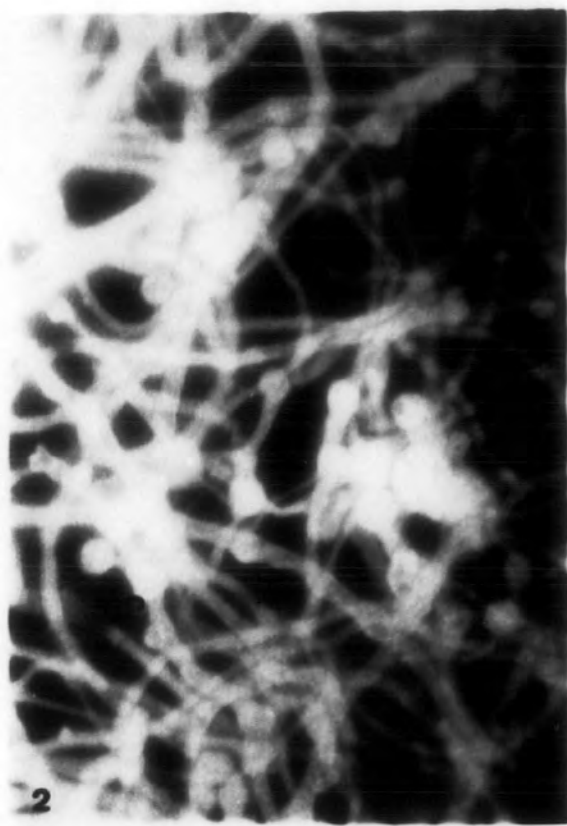
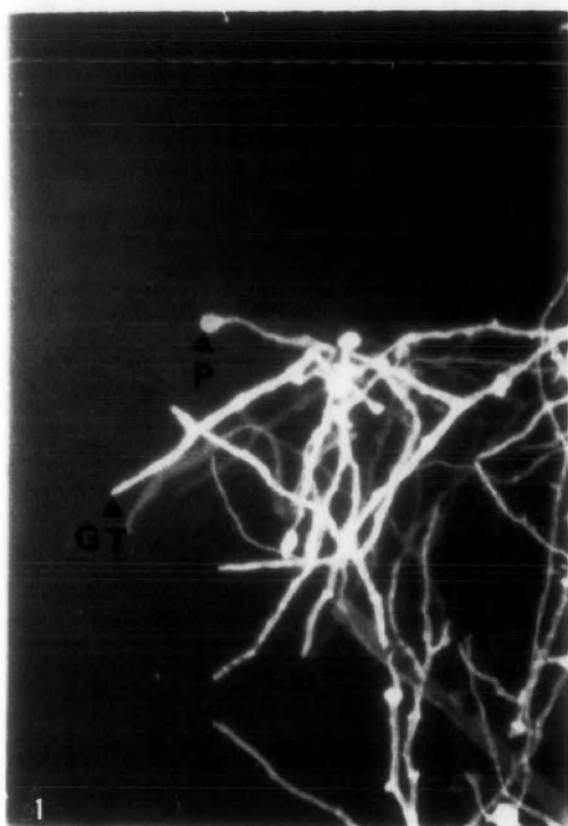


Plate (47)

Regeneration of sporangiophores with basal ends immersed  
in

1. Nitrogen rich medium

magnification X1225

2. Carbon rich medium

magnification X1225

3. Nitrogen rich medium

magnification X3125

4. Carbon rich medium

magnification X3125

Sporangiophores were fixed in acetic acid-alcohol  
and stained with acridine orange

B: Basal end

H: Hyphae

N: Nuclei

Spph: Sporangiphore



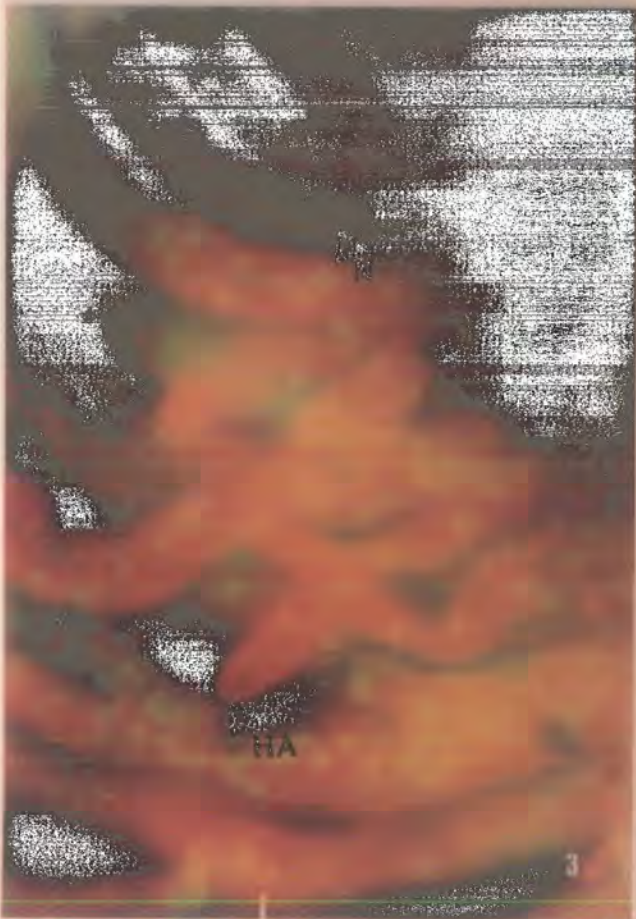


Plate (48)

Regeneration of protoplasts in nitrogen rich medium, showing more branching of developed hyphae. Protoplasts were stained with Uvitex BOPT

- 1 - At 6 hours
- 2 - At 8 hours
- 3 - At 10 hours
- 4 - At 12 hours

magnification X1800

P: Protoplast

H: Hyphae



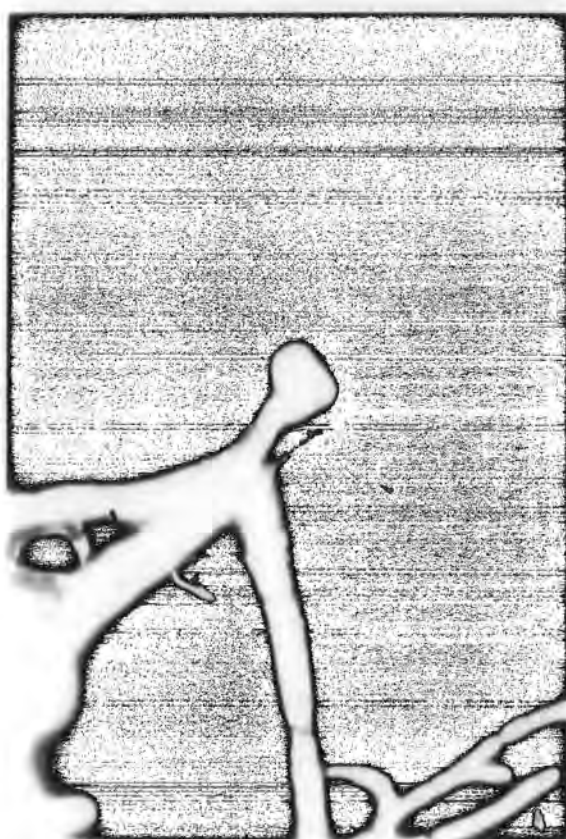
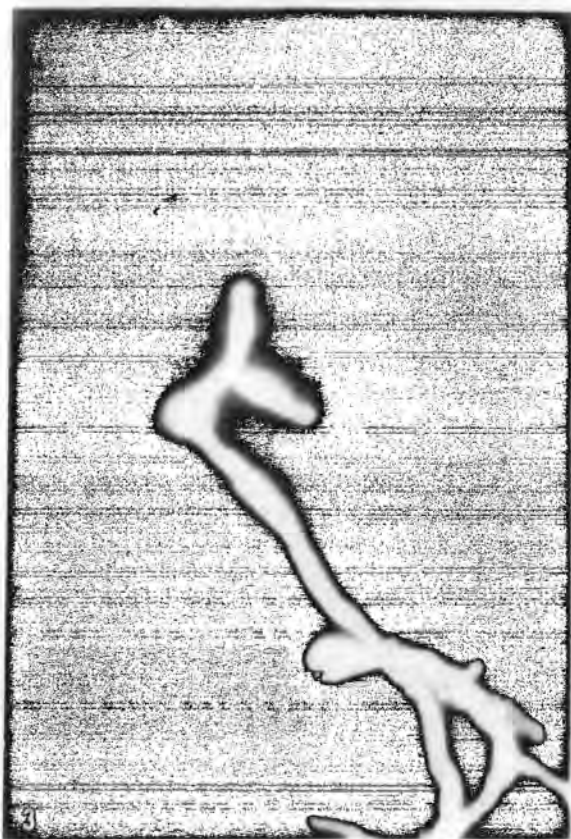
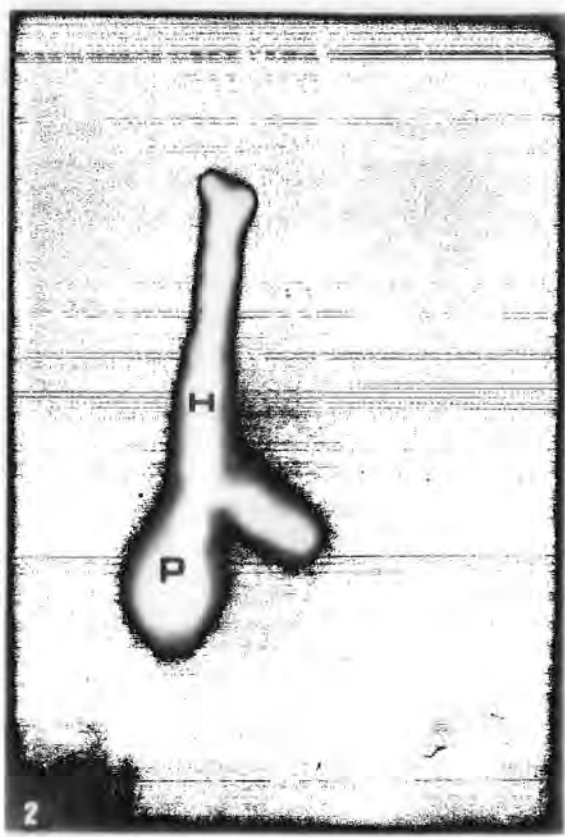


Plate (49)

Regeneration of protoplasts in carbon rich medium,  
showing less branching of developed hyphae. Protoplasts  
were stained with Uvitex BOPT

- 1 - At 4 hours
- 2 - At 5 hours
- 3 - At 10 hours
- 4 - At 12 hours

magnification X1600

P: Protoplast

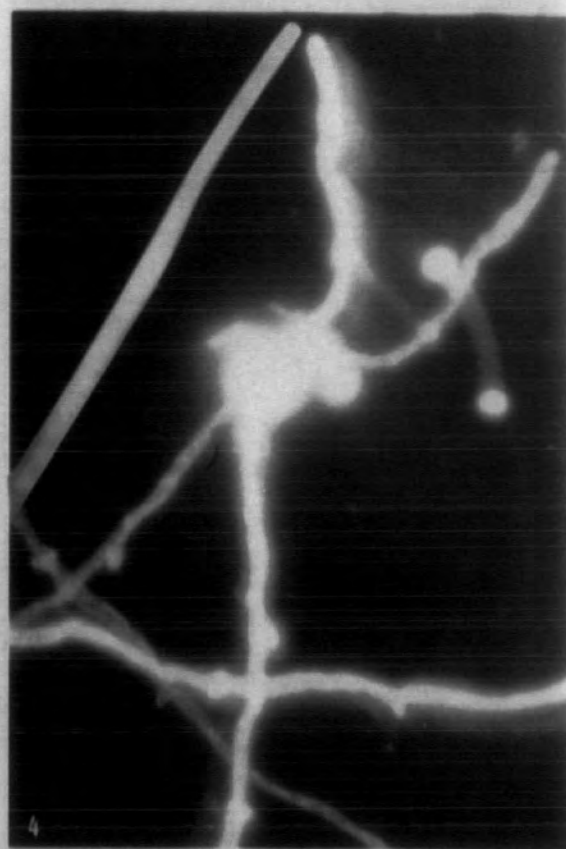
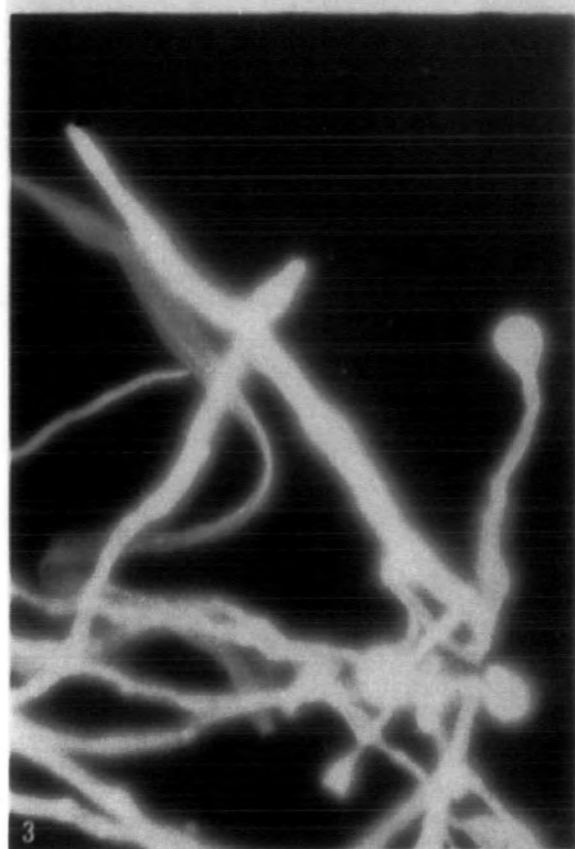




Plate (50)

Granular regeneration from homogenized protoplasts

1. On solid medium

magnification X400

2. In liquid medium

magnification X400

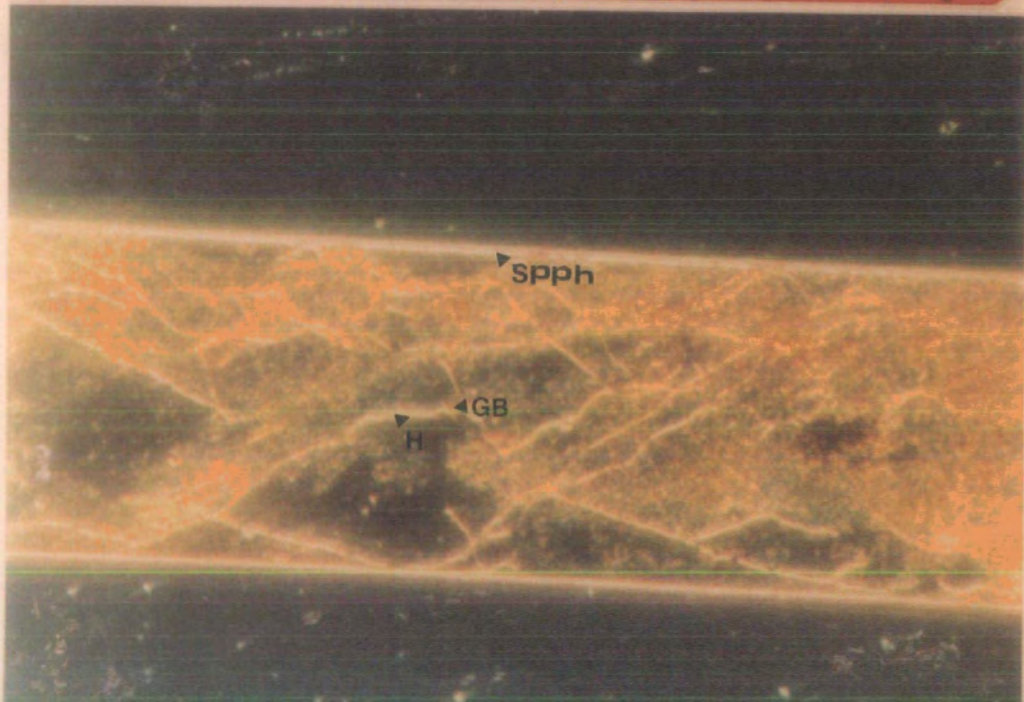
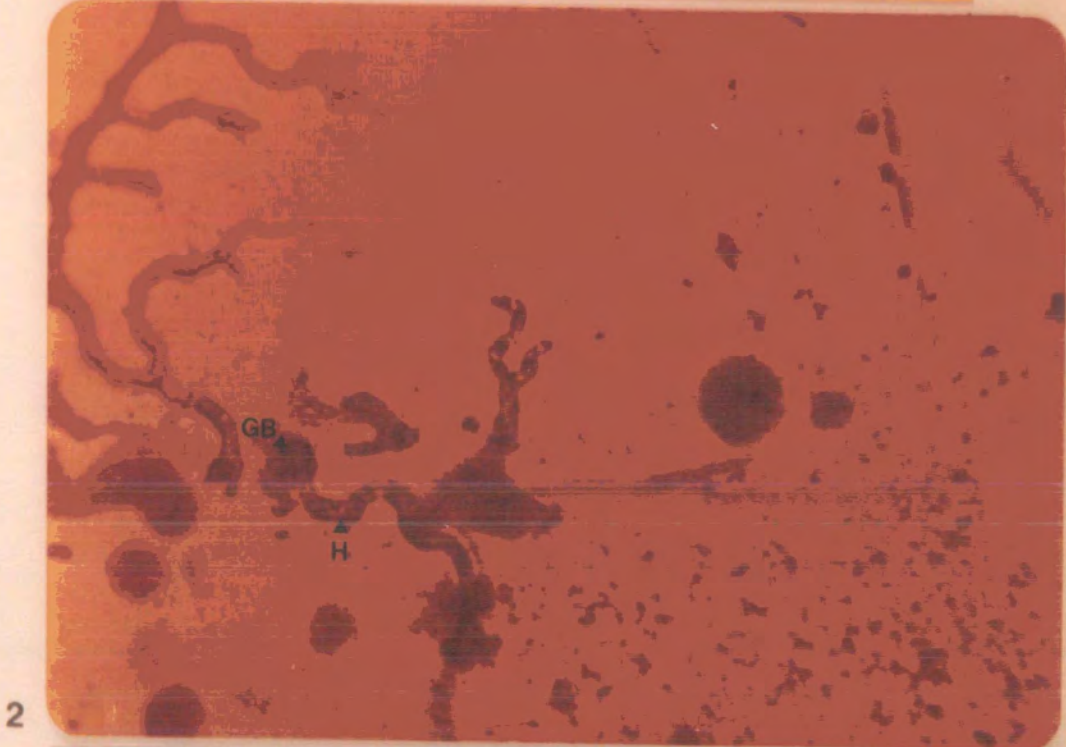
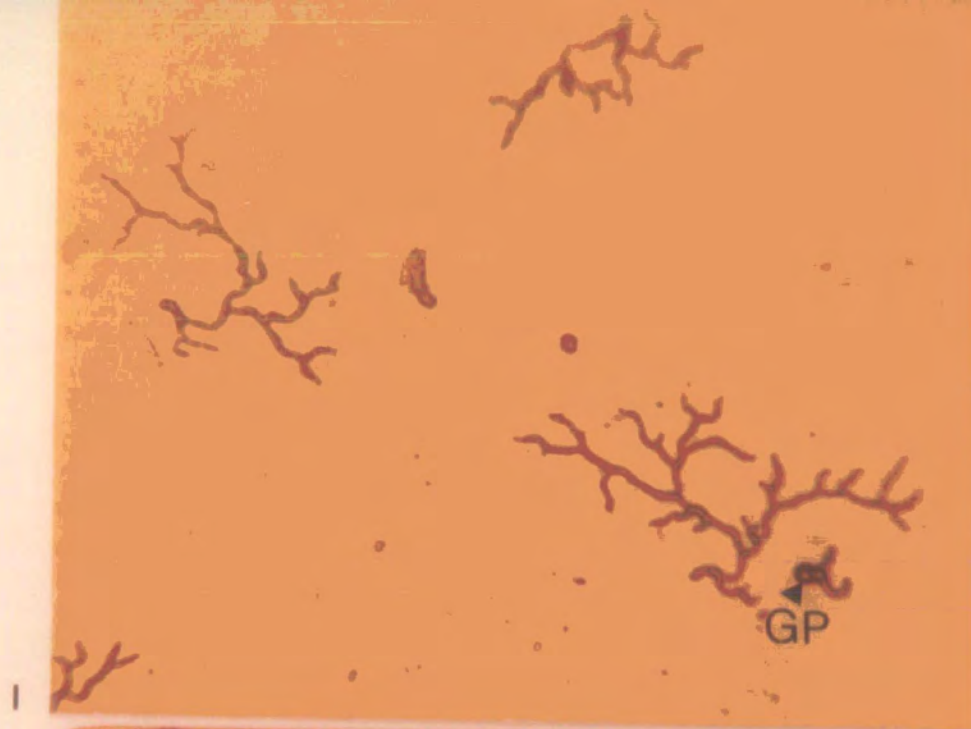
3. Regeneration from granular like structure  
inside the cultured sporangiophore in  
liquid medium

magnification X2800

GB : Granular Body

H : Hyphae

Spph : Sporangiophore



CHAPTER III

RNA and Ribosomal Study

#### 24. INTRODUCTION III

As shown previously in Chapters I and II, the location of RNA, DNA and protein appeared to play a part during growth and development of the sporangiophore. The regeneration of sporangiophores has been shown to be associated with the levels of RNA and protein recovered. RNA and protein contents increased first, followed by DNA. This is in agreement with the results obtained by Van Assche & Carlier (1973) and accordingly they classified Phycomyces as belonging to category I fungi (reviewed by Bramble et al., 1978).

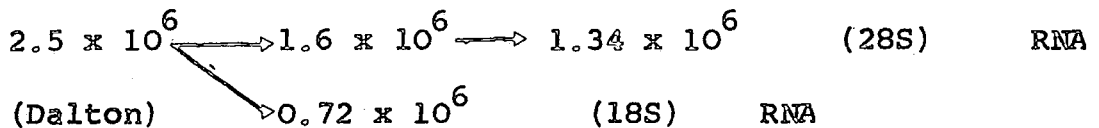
Priority of synthesis of RNA and protein during morphogenesis also resembled that found in Fusarium solani (Cochrane et al., 1971), Lenzites saepiaria (Scheld & Perry, 1970), Microsporium gypsym (Barash et al., 1967), Neurospora crassa (Bhagwat & Mahadevan, 1970), Peronospora tabacina (Hollomon, 1969) and Rhizopus stolonifer (Van Etten et al., 1974). Maximal recovery of these macromolecules was shown to be correlated with growth and development of sporangiophores especially with apical growth. Studies on the qualitative changes in the RNA and ribosome contents, therefore, possibly could give more information about protein synthesis during the growth of asexual reproductive structures of Phycomyces.

Also it was considered to be a useful system for showing if there were any changes in a definite species of RNA, during the regeneration and development, since it has been found that the isolated sporangiophore behaves as an attached sporangiophore. An increase in polysome content of tissues has been regarded as a reflection of increased m-RNA synthesis and hence protein synthesis (Pearson, 1969; Spirin & Gavrilova, 1969; Smith, 1976). Such changes in RNA and of formation of polysomes were followed during the regeneration and growth of different stages of the sporangiophore.

Sporangiophore growth and development has been given relatively little attention in the field of biochemical investigation compared with studies on growth and development of spores and mycelium (Gamow & Prescott, 1972; Van Assche & Carlier, 1973; Pearson & Thomas, 1976). Nucleic acid and protein synthesis during growth and development of filamentous fungi was extensively reviewed by Lovett (1975), Smith & Berry (1975), Berry & Berry (1976), Bull & Trinci (1977), Maclean (1977), Bramble et al., (1978).

Like other eukaryotic cells, it has been established for Phycomyces (Pearson & Thomas, 1976) that the synthesis of the major cytoplasmic and RNA components proceeded initially through the transcription of a large precursor

molecule (Leaver & Key, 1970; Udem & Warner, 1972; Warner, 1974; Lewis, 1974, 1976; Davidson, 1976). The initial precursor, which appears to vary in size from organism to organism, is further processed by cleavage to give a number of intermediates which in turn give rise to the major RNA molecules of mature ribosome (Ingram, 1972). In Phycomyces, on evidence presented by Pearson & Thomas (1976) it is proposed that the major ribosomal RNA (28S and 18S) synthesis, proceeds through the following sequence of molecules:



According to work in higher plants, 28S and 18S RNA were found to be present in 60S and 40S sub-units of eukaryotic cells (Spirin & Gavrilova, 1969; Hadholov & Nikolaev, 1976). In addition, 5S and 5.8S RNA (molecular weight of 40,000 and 60,000 daltons respectively) are also found in the ribosome of eukaryotes. It has been suggested that 5S is present at the surface of 60S sub-unit and may be in contact with the surface of the 40S sub-unit. 5.8S RNA has been found associated with 28S RNA in the 60S sub-unit (Spirin & Gavrilova, 1969; Payne & Dyer, 1971, 1972; Harvey, 1973; Strangeway, 1977; Berry & Berry, 1976; Cox, 1977; Bramble et al., 1978; Eckert et al., 1978). As yet the precursor sequence for 5S and 5.8S RNA has not been resolved for eukaryotic cells (see also Tønnesen, 1978).

Any change in the complement of the RNAs species may be a reflection of the physiological state of the organism. Such changes have been seen as the synthesis of a new RNA species during the sporulation of Saccharomyces cerevisiae (Udem & Warner, 1972; Hopper et al., 1974; Wejksnora & Haber, 1978). Transcription of new mRNA during photo-induced sporulation of Trichoderma viride was found by Stavy et al., (1970). In contrast, other fungi show apparent pre-existing RNA during growth and development (see review by Hadholov & Nikolaev, 1976; Bramble et al., 1978).

Continuous synthesis of tRNA and other species has also been reported to be required during the sporulation of fungi (Burke et al., 1972; Cheng-Shong & Van Etten, 1972). In Phycomyces and other fungi, Burke et al., (1972) Timberlake et al., (1972), Fährnich (1974a,b), Berry & Berry (1976) reported that the sporulation stage could be induced by starvation with concomitant turnover of both RNA and protein. In addition Timberlake et al., (1972) found that during the period of starvation a large proportion of the amino acids which were released by degradation were re-utilized during the growth of the following stages. Degradation of RNAs during the starvation and ageing of fungi is reviewed by Fencel (1978).

Utilization of degraded RNA (ribonucleoside monophosphate) as biosynthetic precursors for synthesis of both nucleic acids and other cell materials and also catabolism, the ribose portion for energy purposes has been reported for Bdellovibrio bacteriovorus (Hespell & Mertens, 1978).

Degradation of polysomes during starvation and thus alteration of protein synthesis has also been reported to occur during starvation and ageing of both eukaryotic and procaryotic cells (see Storck & Wimmer, 1975; Mariotti & Ruscitto, 1977; Mirkes, 1977; Balland et al., 1978).

Degradation of RNA and ribosomes during ageing and autolysis was found by Righelato et al., (1968); Nagazaki (1968b).

Trinci and Righelato (1970) found that the nuclei also became less electron dense in association with the ribosomal degradation of the starved cells. Additionally the organization of nucleic acids and protein in the growth and development of plants in general is extensively reviewed by Bogorad & Weil (1977).

The importance of the translation stage in protein synthesis and wall synthesis during growth and development of Phycomyces has been suggested by Van Laere et al., (1976, 1977). Since the growth and development of Phycomyces, spores was inhibited in the presence of cycloheximide and 5-fluorouracil analogue enzymes required for cell wall



synthesis appear to be under control at the translational level, possibly through the existence of preformed mRNA. Additionally sporulating cells of Bacillus subtilis have been shown to contain mRNA for both sporulating and vegetative cells (Fukuda & Doi, 1977).

Dissociation and reassembly of the polyribosome has been shown to be associated with activity of the RNA and protein synthesis in living systems (Lin et al., 1967; Adelman & Lovett, 1972; Harvey, 1973; Adelman & Lovett, 1974a; Burdon, 1976; Cox, 1977). A shift of monosomes to polyribosome fractions were found associated with the gametangia formation of Allomyces arbuscula (Fähnrich, 1977). Increased recovery of polysomes were also found to be required during a differentiation stage of Blastocladiella emersonii. Botryodiplodia theobromae, Neurospora crassa (Henny & Storck, 1963a,b; 1974; Bramble & Van Etten, 1970; Adelman & Lovett, 1974b). Likewise the regeneration of sphaeroplasts of Saccharomyces cerevisiae was shown to be associated with the recovery of membrane bound ribosomes, as have other stages in fungal development (reviewed by Berry & Berry, 1976). The presence of more membrane-bound ribosomes at the tip of the sporangiophore of Phycomyces (Jan, 1974) appeared to be specifically associated with a special local differentiation for

sporulation. Similarly abundant endoplasmic-mRNA has been found associated with the formation and germination of spores of Peronospora tabacina (Hollomon, 1969, 1970, 1971, 1973) and of Neurospora crassa (Mirkes, 1974). A shift of protein synthesis from cytoplasmic to membrane fractions during spore formation of Bacillus subtilis was also found by Goldman (1976). Turnover of membrane-bound ribosomes have recently been shown to be associated with the late stage of morphogenesis, while the turnover of membrane-free ribosomes was shown to be associated with early growth and differentiation of Dictyostelium discoideum (Bourguignon & Katz, 1978). Activity of chitin synthase was found to be more in the microsomal fraction of Mucor rouxii (McMurrough & Bartnicki-Garcia, 1971). In this study an investigation was made into the changes in specific RNA and ribosome fractions during the differentiation and growth of the sporangiophore and protoplasts of Phycomyces blakesleeianus.

MATERIALS AND METHODS25. EXTRACTION AND FRACTIONATION OF NUCLEIC ACIDS1. Detergent-phenol extraction

The method described by Kirby (1965) and Loening (1967) and as adapted by Pearson (1969) and Stewart & Letham (1977) was used. Stage I sporangiophores were frozen with liquid air and homogenised with a pestle and mortar, with 5 ml of detergent medium containing:-

1% Tri-isopropyl-naphthalene sulphonate

6% p-aminosalicylate

50 mM NaCl

10 mM MgCl<sub>2</sub>

10 mM Tris-HCl pH 7.4

The mixture of the tissue and detergent medium was mixed with 5 ml of a phenol mixture consisting of:-

Redistilled phenol containing

10% m-cresol

0.1% 8-hydroxyquinoline saturated with 10 mM tris-HCl  
pH 7.4

followed by blending using a 'Whirlimixer' (Fison scientific apparatus Ltd., Loughborough). This mixture was centrifuged for 10 minutes at top speed in an MSE clinical centrifuge or Minor bench centrifuge at room temperature. The lower layer of phenol was removed using a Pasteur pipette and the upper aqueous layer and interphase material was brought

to 0.5M with NaCl. The aqueous solution was extracted a second time by the addition of an equal volume of phenol mixture followed by centrifugation as above. Nucleic acids were precipitated from the aqueous phase by adding 2 volumes of cold absolute ethanol and keeping it overnight at  $-20^{\circ}\text{C}$ . Precipitated nucleic acids were collected by centrifugation at top speed in an MSE bench centrifuge for 10 minutes. 80% ethanol, containing 0.5% SDS, was used to wash the nucleic acid pellet and centrifugation was carried out as previously described. Finally the pellet was washed several times in 70% ethanol containing 0.1M sodium acetate. The RNA pellet was normally redissolved in a small volume of the appropriate electrophoresis buffer and either used immediately or stored at  $-20^{\circ}\text{C}$ . All of the previous steps were carried out at  $0-4^{\circ}\text{C}$  and the various solutions stored at  $4^{\circ}\text{C}$ . Nucleic acid concentrations in solution were measured by the method of Tester & Dure (1966) as described above.

## 2. Polyacrylamide gel preparation

The method of Loening (1967, 1968a) as modified by Pearson (1969) and Strangeway (1977) was used here. The stock acrylamide was prepared as a solution of acrylamide and methylene bisacrylamide recrystallised from chloroform and acetone respectively (Loening, 1967). For gels of an acrylamide proportion of less than 5% a stock monomer



solution of 15% acrylamide and 0.75% bisacrylamide was prepared. For gels of a greater acrylamide percentage the monomer stock solution contained 15% acrylamide and 0.35% bisacrylamide. These stock solutions were stored in the dark at room temperature. Ammonium persulphate (10%) was prepared and made up fresh for each preparation. Stock solutions of buffer were prepared as follows:-

"E" buffer of Loening (1969)

0.036M Tris-HCl pH 7.7

0.03M Sodium dihydrogen phosphate

0.001M Disodium EDTA

This was prepared as a three-fold concentrated stock solution "3E". Polyacrylamide gels were made up as follows:-

|                               | Gel strength % |            |            |
|-------------------------------|----------------|------------|------------|
|                               | <u>2.2</u>     | <u>2.6</u> | <u>7.5</u> |
| Stock acrylamide<br>(monomer) | 3.6 ml         | 4.33 ml    | 12.5 ml    |
| "3E" buffer                   | 8.32 ml        | 8.32 ml    | 8.32 ml    |
| Water                         | 12.78 ml       | 12.11 ml   | 3.94 ml    |
| TEMED                         | 0.02 ml        | 0.02 ml    | 0.02 ml    |
| Ammonium persulphate<br>(10%) | 0.2 ml         | 0.2 ml     | 0.2 ml     |

The mixture was degassed under vacuum before 0.2 ml of 10% ammonium persulphate was added. Polyacrylamide gels were cast from the above mixture in vertically held 'plexiglass' tubes (9 cm x 0.6 cm interior length diameter) to a depth of 8.0 cm. The above volume of polyacrylamide mixture should give enough material for the preparation of 8 gels.

The lower end of each 'plexiglass' tube was sealed with dialysis membrane held in the place with a rubber ring to support the gels. To prevent the solution of the polyacrylamide passing through the dialysis membrane before it had set, a closed rubber tube was fitted over the membrane (forming an air seal) which was removed after the gels set. In the case of the 7.5% polyacrylamide gel the surface of the gel solution was carefully layered with a drop of water applied to the meniscus of the solution to ensure a flat surface. This was not a problem with 2.2% and 2.6% gels. The running buffer was prepared by diluting the stock buffer solution "3E" three-fold and 0.3% of SDS was added to the final concentration.

### 3. Electrophoresis and fractionation

This was carried out using a Vokam power pack, set on constant current made at 5 MA/gel, giving a voltage of 48-50 V. for approximately 30 minutes to pre-run the gels. 25-20  $\mu$ g of RNA in 0.02-0.1 ml of buffer plus a small quantity, 5% of solid sucrose, were loaded onto each gel and the electrophoresis run for the required length of time (2-3 hours). At the end of the run the dialysis membrane was removed and the gels allowed to slide out of the running tubes into a petri dish of water or 7% acetic acid, and were left for 1-2 hours to remove the high absorbance substances from the gels. 2.6% gels were

removed easily, but 7.5% gels were difficult to remove. Thus an injection instrument was modified, to increase the pressure over the surface of the gel, which pushed the gel out of the 'plexiglass' tube.

#### 4. U.v. absorption scanning

To determine the position of the bands of the RNA on the gels, they were scanned in a Joyce-Loebl polyfrac fitted with a 265 nm interference filter. The area under the peak on gel traces was measured by weighing the tracings of the peaks. To estimate RNA for loading, the method of Tester & Dure (1966) was used where  $OD_{260} - OD_{290} = 11 = 0.5 \text{ mg} \cdot \text{ml}^{-1} \text{ RNA}$ .

#### 5. Molecular weight determination

The apparent molecular weights of RNA fractions were determined from a plot of log molecular weight against the mobility of the RNA fraction along the gels (in cm).

E.coli ribosomal RNA species were used as a standard marker for the determinations (see Loening, 1968b & c, 1969; Bishop et al., 1967; Pearson, 1969; Strangeway, 1977).

#### 6. Extraction and fractionation of ribosomes

The method of Jachymczyk & Cherry (1968) as described by Pearson (1969) and Strangeway (1977) was used to extract polyribosomes from the sporangiophore of Phycomyces.



Washed stage I (1 gm fresh weight) sporangiophores were frozen in liquid nitrogen and were homogenized to a powder in a precooled mortar. Addition of 3 ml.gm<sup>-1</sup> of the following extractant buffer was made:-

0.02 M Tris-HCl pH 8.0

0.25 M Sucrose

0.01 M Magnesium chloride

0.015 M Potassium chloride

0.005 M B - mercaptoethanol

0.5% Sodium deoxycholate

The homogenate was centrifuged at 12,000 x g for 20 minutes at 5°C in an MSE 'high speed' 18 centrifuge. Using a sterilized pipette, the post-mitochondrial supernatant was removed and layered over 1 ml of 0.5 M and 3 mls of 0.5 M sucrose; both made up in 0.02 M tris-HCl pH 8.0, 0.01 M magnesium chloride, 0.015 M potassium chloride and 0.005 M B - mercaptoethanol. This was followed by centrifugation at 40,000 x g. for 3 hours at 0°C in a 10 x 10 ml titanium angle rotor fitted in an MSE superspeed 65 ultracentrifuge. All extraction and transfer steps were carried out at 4°C to maintain polyribosomes intact and prevent their degradation. The high speed supernatant was decanted and the tube drained by inversion. The tubes were also wiped with an absorbant tissue paper. Sedimented polyribosomal pellets were used immediately or stored at -20°C.



### Spectrophotometric estimation of ribosomes

Polyribosome pellets were dissolved in the minimum amount of the appropriate buffer which contained 10% sucrose. 0.01 ml of the polyribosome solution was diluted in 1 ml of distilled water in 1 ml cuvette. The absorbance of the polyribosomes was measured in the Unicam 1800 spectrophotometer. Maximum absorbance was found at 260 nm wavelength. In this case, the ribosomal RNA was estimated from the u.v. absorption spectrum using the Tester and Dure (1966) method, where  $OD_{260} - OD_{290} = 11 = 1.0 \text{ mg} \cdot \text{ml}^{-1}$  ribosomes.

### Preparation of gel electrophoresis and fractionation of ribosomes

The method used was essentially that of Strangeway (1977). 2.2% polyacrylamide gels were made as previously described. Five-fold concentrations stock solutions of the following buffer were employed (Loening, 1968a and Strangeway, 1977):

0.1 M Tris-acetate pH 8.0

75 mM Potassium acetate

37.5 mM Magnesium acetate

Gels were pre-run for 30 minutes at 13 V per gel (104 V for 8 gels) using a Vokam power pack set on a constant voltage mode. Solutions of up to 40  $\mu\text{l}$  of polyribosomes in the electrophoresis buffer plus 10% sucrose containing

approximately 15 µg ribosomes were loaded carefully onto the gels. Electrophoresis was carried out for 2 hours at 2°C in a cold room with buffer circulation between the two reservoirs. Following electrophoresis, the gels were washed in 7% acetic acid for 1-2 hours. Separated bands of the ribosomes were seen after the wash as white precipitated bands, and were also visualized clearly after staining for RNA with 0.02% toluidine blue (destaining with several changes of distilled water) and by staining for protein with 0.1% amido black in 7% glacial acetic-acid (destaining with several changes of 7% glacial acetic-acid). Washed gels, without staining, were scanned at 265 nm in Joyce Loebel Polyfrac.

#### 7. Extraction of RNA from isolated ribosomes

The method described by Strangeway (1977) was used. Solutions containing approximately 30 µg ribosomes (15 µg RNA) for 2.6% polyacrylamide gels and 60 µg ribosomes (30 µg RNA) for 7.5% gels were loaded onto the gels which were electrophoresed in the ribosomes running buffer plus 0.3% SDS. Gels were treated as for RNA fractionations as already described.

#### 8. Determination of polyribosomes presence

An equal amount of the ribosomal solution in the electrophoresis buffer containing 10% sucrose was mixed

with an equal amount of  $10 \text{ mg} \cdot \text{ml}^{-1}$  RNAase,  $40 \text{ } \mu\text{l}$  of the solution containing approximately  $15 \text{ } \mu\text{g}$  ribosomes were loaded as described above.

### CHAPTER III

#### Results

## 26. QUALITATIVE STUDIES OF RNA OF SPORANGIOPHORES

### 1. Characterization of the method

Total RNAs of stage I sporangiophores (10-20 mm in length) were extracted by the phenol-detergent method. The absorption spectrum is shown in Figure (28). As shown, the maximum and minimum absorption were at 260 nm and 240 nm respectively.

The ratios of  $\frac{E_{260}}{E_{240}}$  and  $\frac{E_{260}}{E_{280}}$  values were as follows:-

|      |      |
|------|------|
| 1.58 | 1.92 |
|------|------|

This indicated essentially the absence of any contamination or interference with extraneous substances and also acted as an indication of the purity of RNA (see Peterman, 1964; Tester & Dure, 1966; Pearson, 1969; Strangeway, 1977).

### 2. RNA fractionation

RNA's species were fractionated by 2.6 and 7.5% polyacrylamide gel electrophoresis system. Traces for high and low molecular weights RNA are shown in Figure (29). Using highly polymerized E.coli RNA as a standard, a linear relationship between the mobility and log. molecular weight for electrophoretically separated ribosomal RNA (rRNA) existed, Figure(30), see Bishop et al., (1967), Loening (1968c), Strangeway (1977). Sedimentation values of 28S and 18S were obtained for the high molecular weight

Figure (28)

U.v. absorption spectrum of RNA extracted from  
sporangiophores



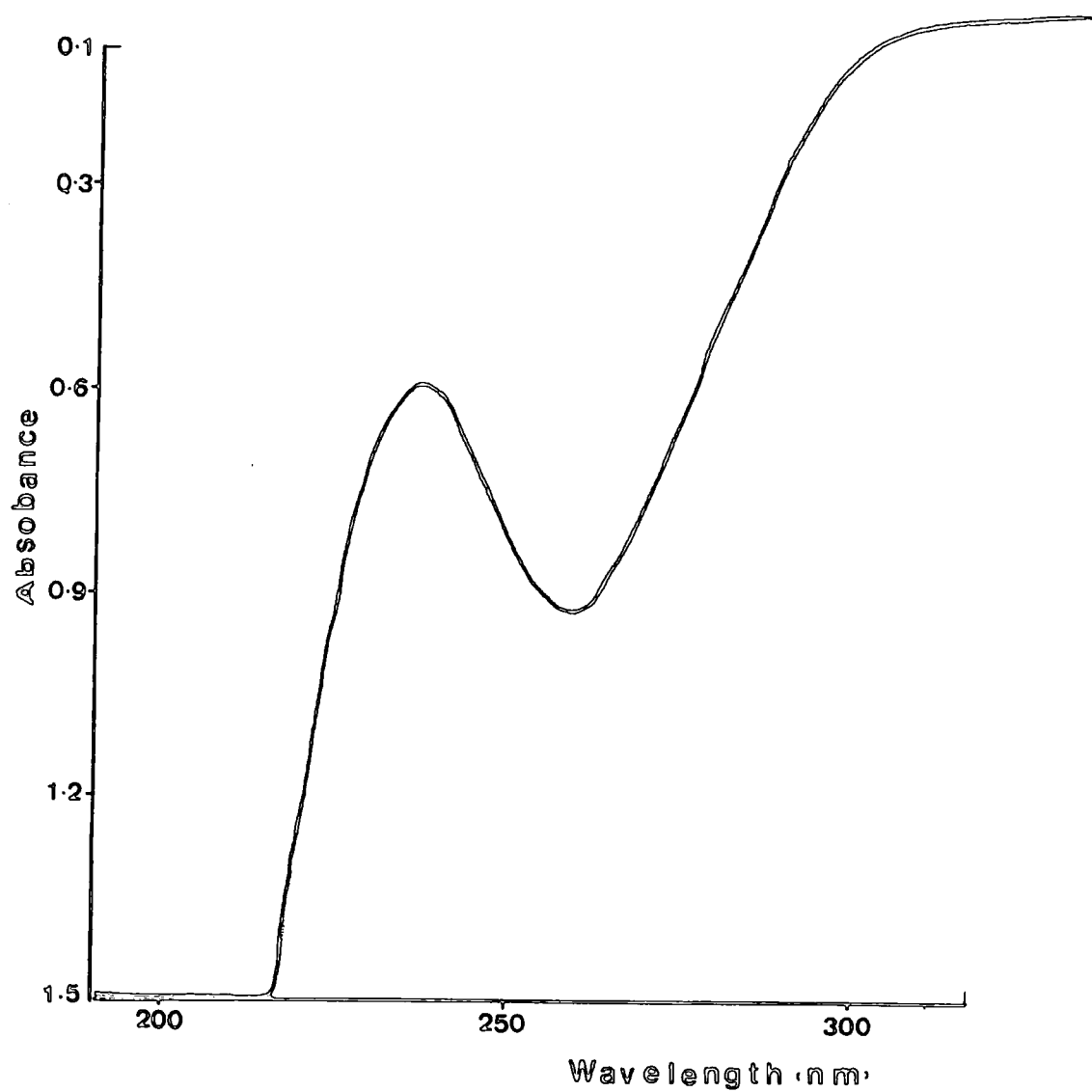


Figure (29)

PAGE of high and low molecular weight RNA species extracted from full length sporangiophores

2.6% gel

A. High molecular weight rRNA

1-18S rRNA

2-28S rRNA

B. High molecular weight rRNA loaded with E.coli RNA

1-16S of E.coli

2-18S of Phycomyces

3-26S of E.coli

4-28S of Phycomyces

7.5% gels

C. Non treated RNA

1-4S

2-5S

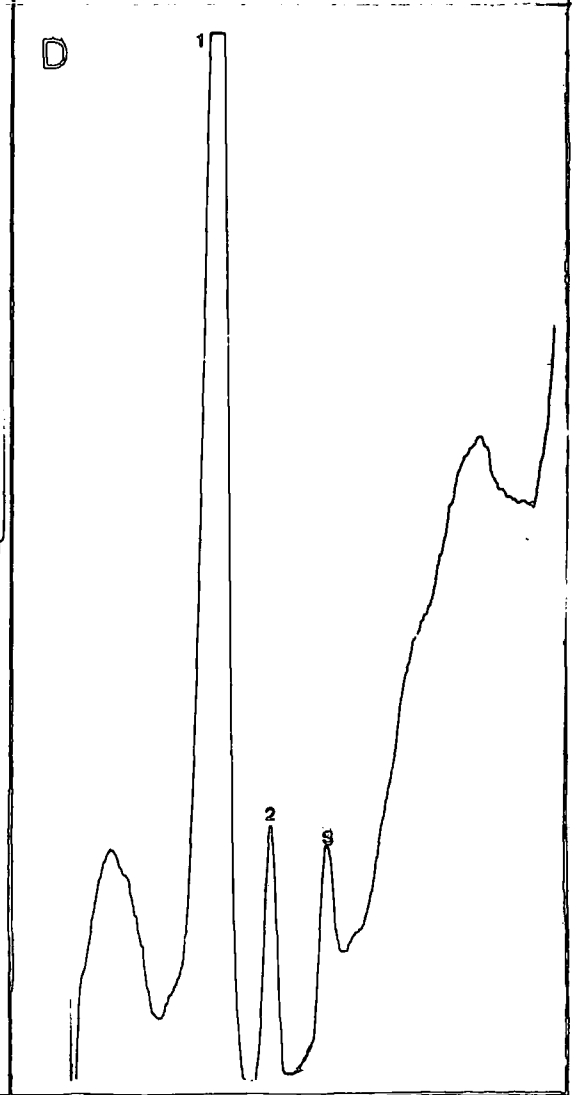
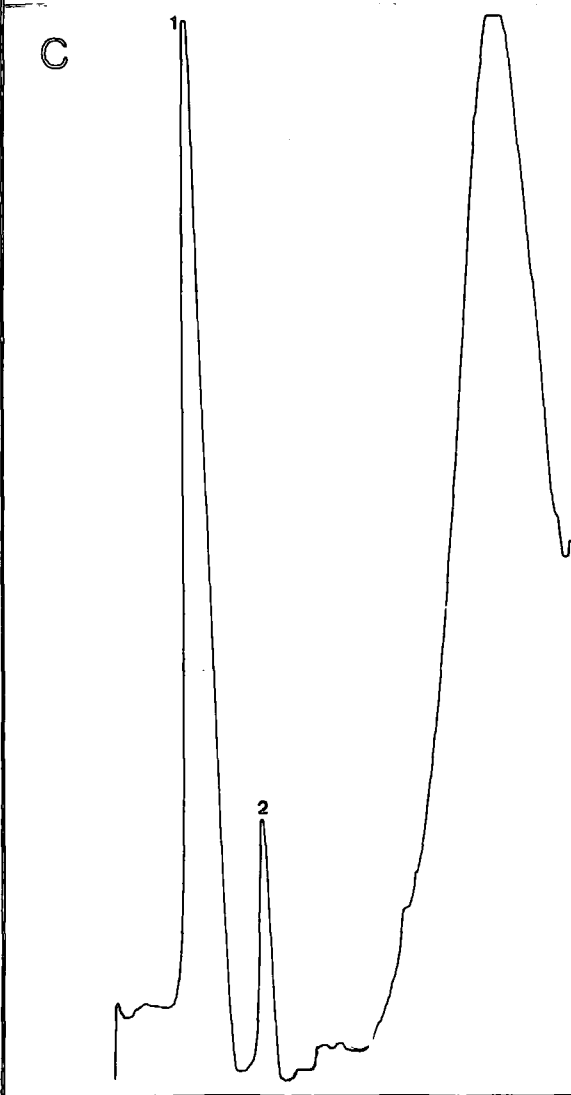
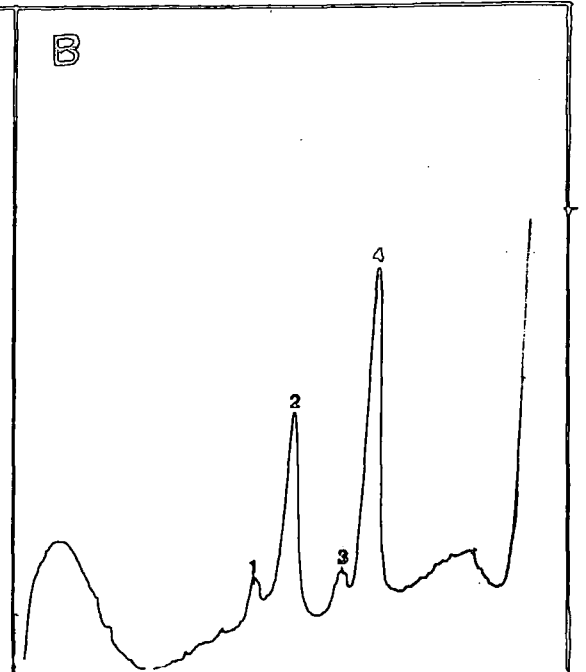
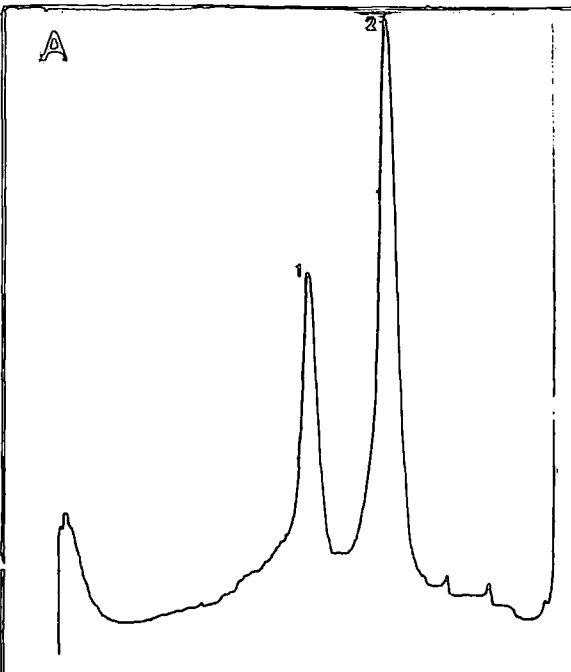
D. Heat treated RNA

1-4S

2-5S

3-5.8S

A 265 nm

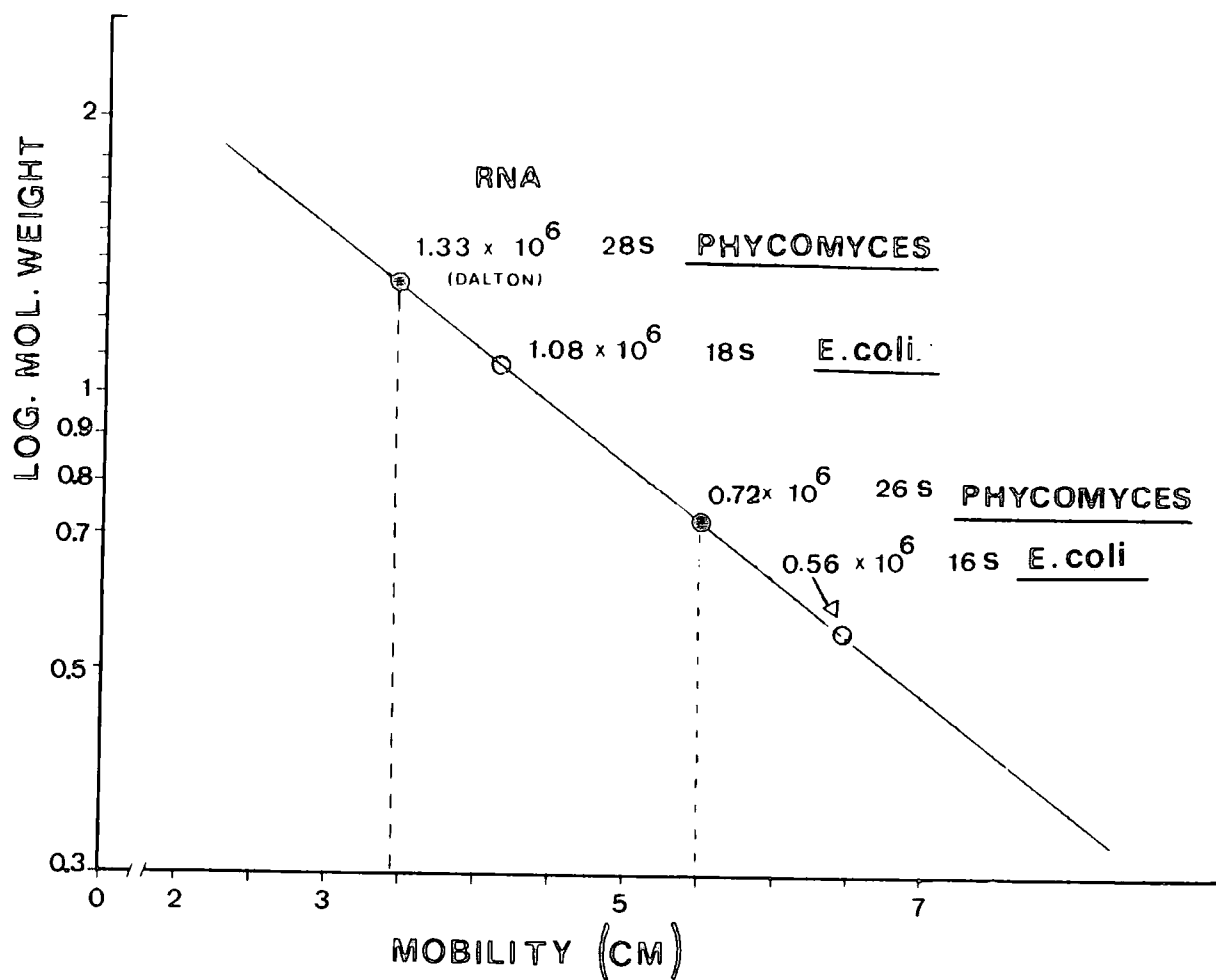


+Ve

-Ve

Figure (30)

Mobility and molecular weight of RNA extracted from the sporangiophore. RNA of E.coli was used as a standard.



ribosomal RNA. These values resembled those found for mycelium by Pearson & Thomas (1976) who determined apparant molecular weight of 1.34 and  $0.72 \times 10^6$  dalton respectively.

Identification of 5S and 4S fractions for sporangio-  
phore was made by comparison with mycelial low molecular  
weight RNA, Figure (31C).

### 3. RNA extraction and fractionation of tip, middle and basal segments

Using similar systems as described above, total RNA  
were also extracted and fractionated from tip, middle and  
basal segments of stage I sporangiophore. These segments  
were equal in length and fresh weight. U.v. absorption  
spectra, Figure (32), showed a maximum and minimum absorption  
at 260 and 240 nm wavelength respectively. The ratios  
of  $\frac{E_{260}}{E_{240}}$  and  $\frac{E_{260}}{E_{280}}$  values are shown in Table (10)

The molecular weight values were the same for RNA  
extracted from tip, middle and base segment and also  
resembled those found for RNA extracted from sporangiophores  
which were in full length as described above.

Using the Tester & Dure (1966) equation (see materials  
and methods) total concentrations of RNA were calculated:  
a greater concentration of RNA,  $550 \mu\text{g RNA} \cdot \text{gm}^{-1}$  fresh weight

Figure (31)

PAGE of high and low molecular weight RNA species  
extracted from mycelium

2.6% gels

A. Non-treated RNA

1-18S rRNA

2-28S rRNA

3-DNA

B. Heat-treated RNA

7.5% gels

C. Non-treated RNA

1-4S RNA

2-5S RNA

D. Heat-treated RNA

1-4S RNA

2-5S RNA

3-5.8S RNA

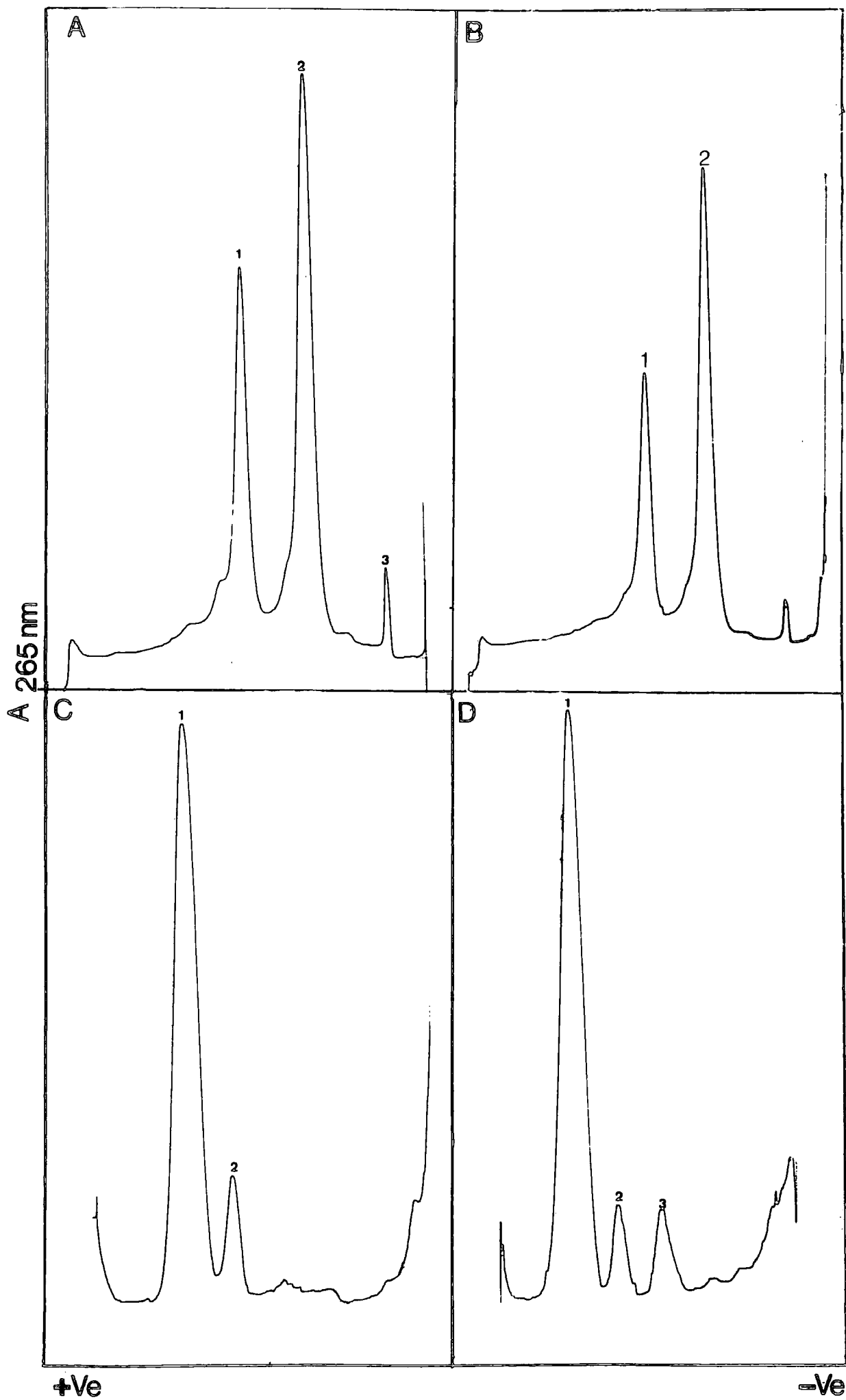




Figure (32)

U.v. absorption spectra of RNA extracted from tip,  
middle and basal segments

T - Tip

M - Middle

B - Base

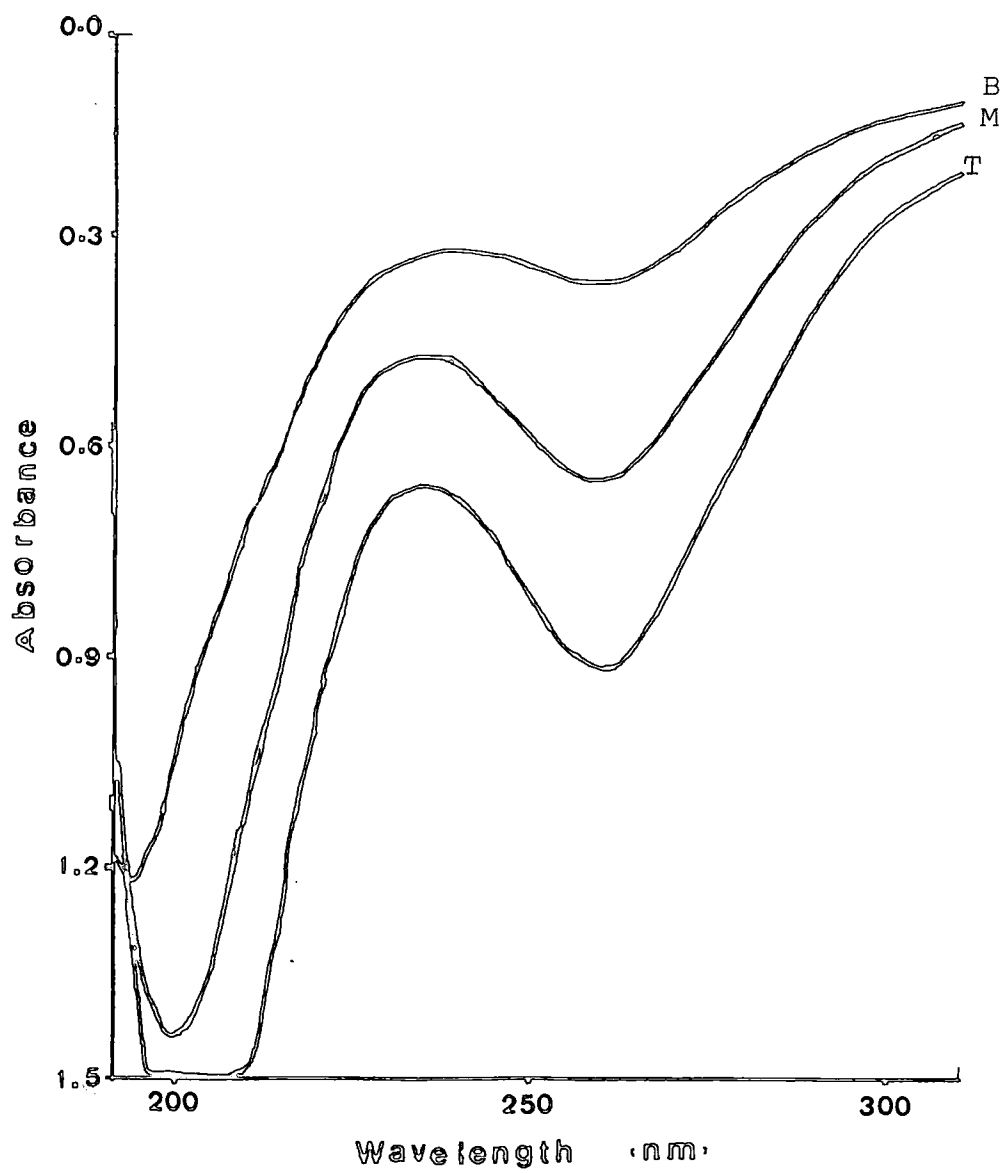


Table (10)

The ratios of  $\frac{E260}{E240}$  and  $\frac{E260}{E280}$  of RNA extracted from tip,

middle and basal segments

| <u>Segments</u> | $\frac{E260}{E240}$ | $\frac{E260}{E280}$ |
|-----------------|---------------------|---------------------|
| Tip             | 1.35                | 1.45                |
| Middle          | 1.23                | 1.32                |
| Base            | 1.19                | 1.37                |

tissue was found for tip segments compared with 235 and 163  $\mu\text{g}\cdot\text{gm}^{-1}$  obtained for middle and basal segments respectively. Such decline in the total concentration of the RNA, from tip to basal regions, is in agreement with the observations which were obtained and reported during the cytological and quantitative studies (see Chapter I and II) where the tip region showed a great quantity of RNA.

### 1. Distribution of RNA fractions

Quantitative measurements of the fractionated RNA species which were extracted from different regions, were obtained by weighing the trace area of each fraction (for example area for each 28S, 18S, 5S and 4S). The area of each peak (measured as weight) was expressed as a percentage of total peak area. The area of each of 28S and 18S RNA for the tip, middle and basal segments was measured from traces in Figure (33), while in Figure (34) are shown the area of 4S and 5S of similar segments. The results are shown in Table (11). Virtually no change in specific fraction was found except 28S rRNA fractions showed a slight decline in proportion but this may not be significant.

Figure (33)

PAGE of high molecular weight rRNA species extracted  
from tip, middle and basal segments

2.6% gels

A. Tip

1 - 18S rRNA

2 - 28S rRNA

B. Middle

18S rRNA

28S rRNA

C. Base

18S rRNA

28S rRNA

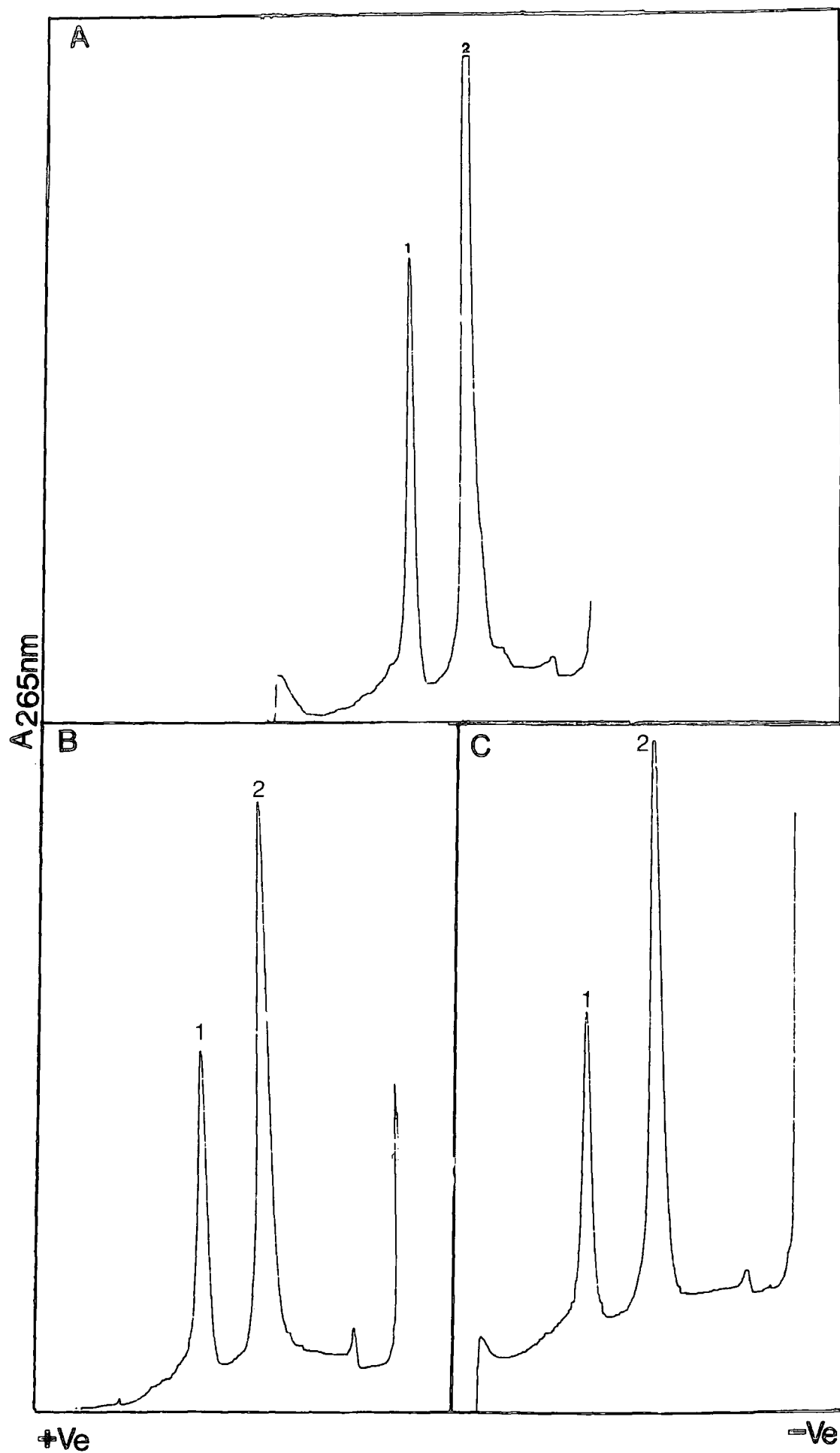


Figure (34)

PAGE of low molecular weight RNA species extracted from  
tip, middle and basal segments using 7.5% gels

Tip

A. Non-treated RNA

1-4S

2-5S

B. Heat-treated RNA

1-4S

2-5S

3-5.8S

Middle

C. Non-treated RNA

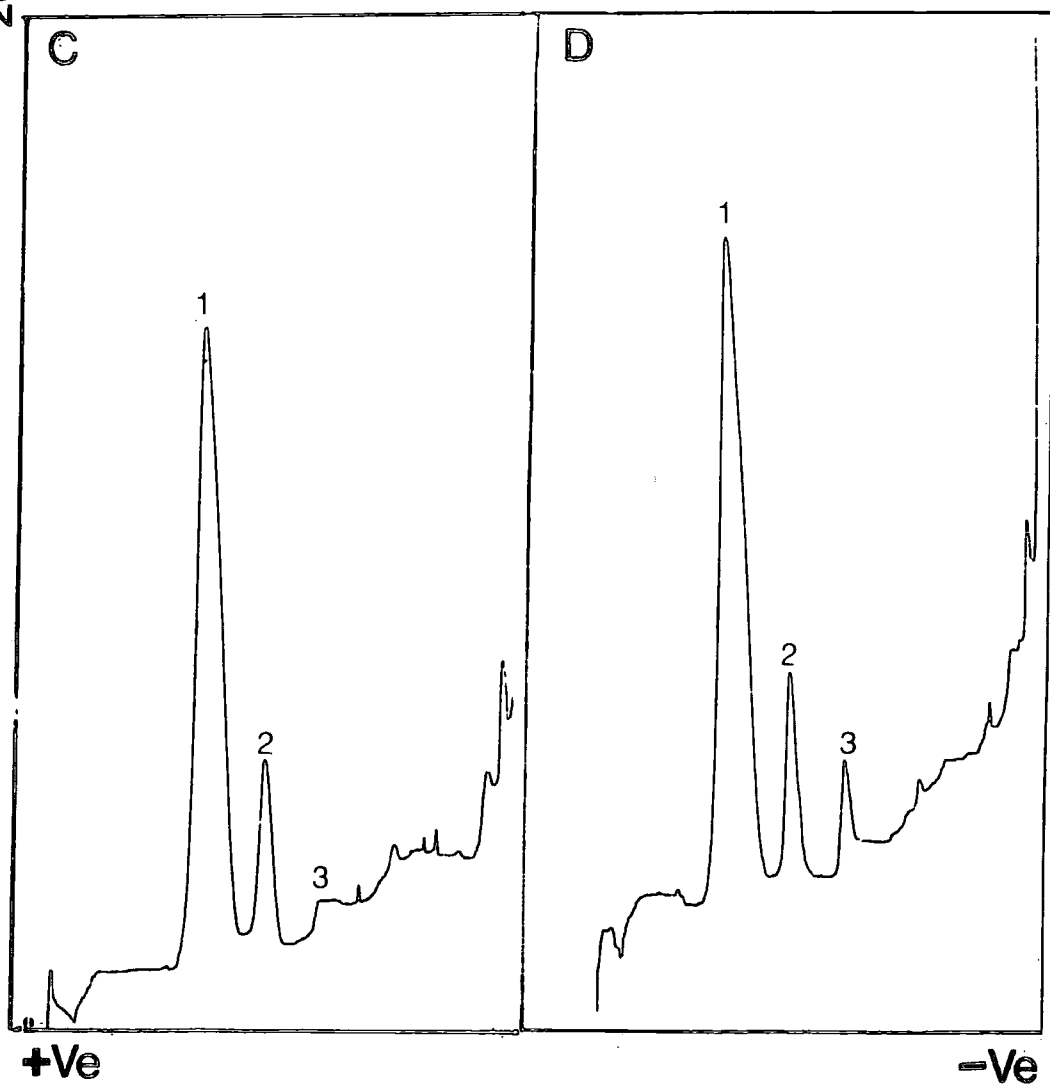
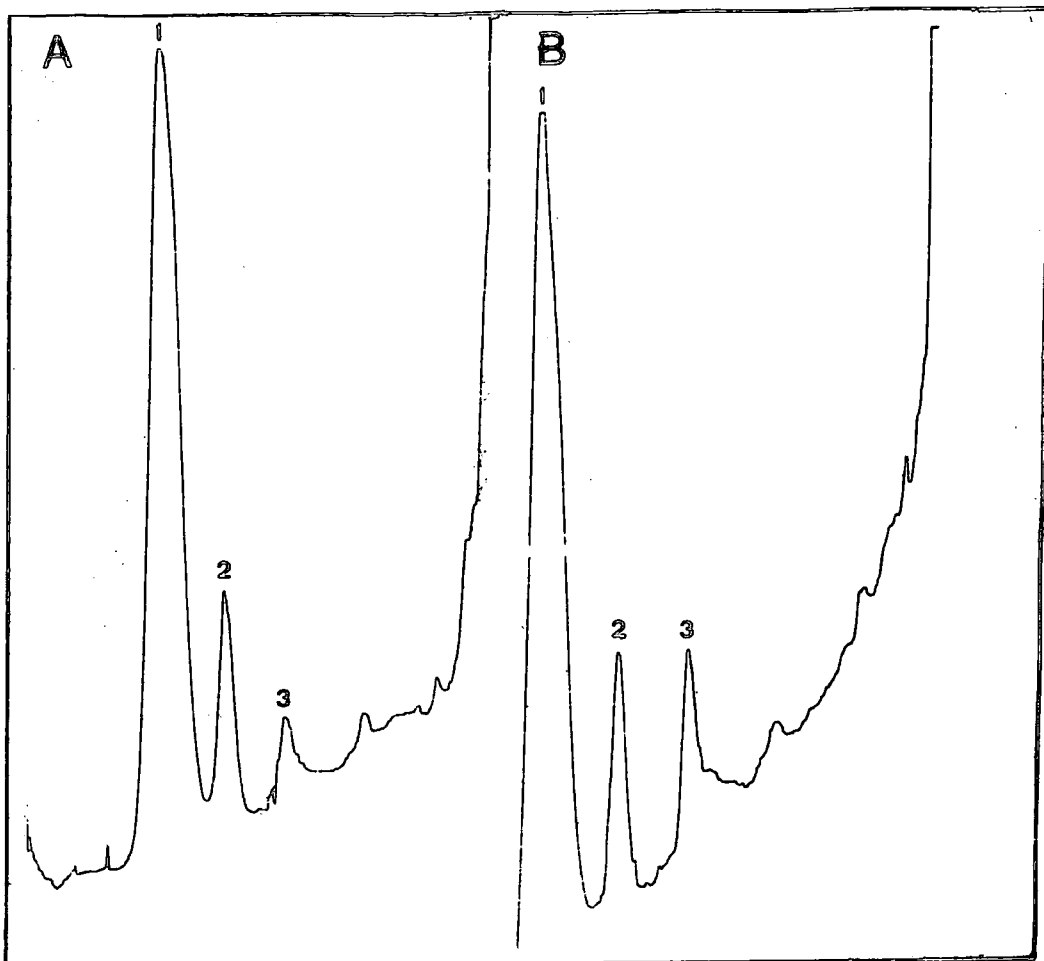
D. Heat-treated RNA

Base

E. Non-treated RNA

F. Heat-treated RNA

A 265 nm





A 265 nm

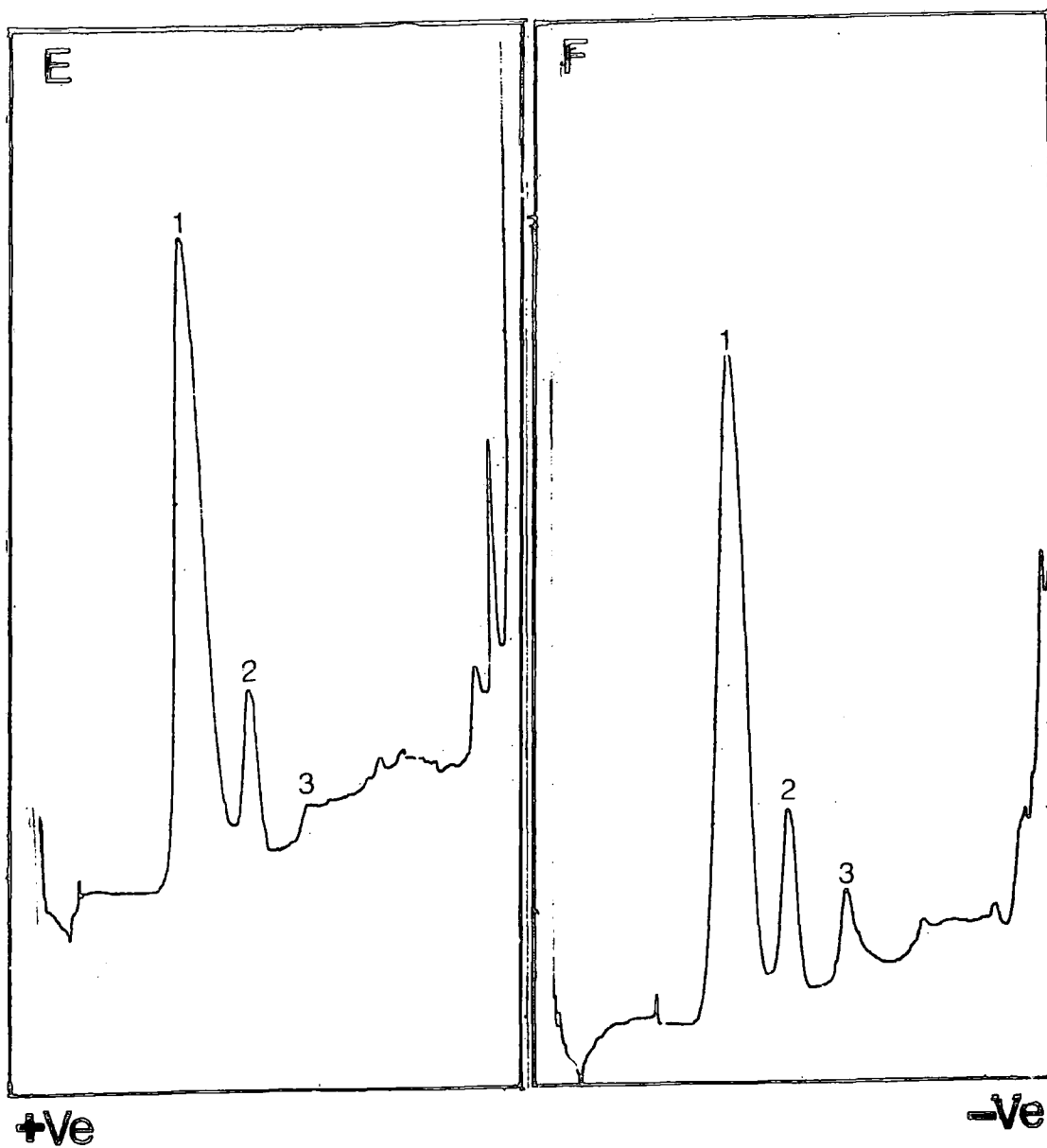


Table (11)

Percentage of total gel RNA fractionations, extracted from tip, middle and basal segments.

| <u>RNA fraction</u>              | <u>% of total gel RNA</u> |               |             |
|----------------------------------|---------------------------|---------------|-------------|
|                                  | <u>Tip</u>                | <u>Middle</u> | <u>Base</u> |
| <u>High molecular weight RNA</u> |                           |               |             |
| 28S                              | 71                        | 67            | 64          |
| 18S                              | 28                        | 32            | 35          |
| <u>Low molecular weight RNA</u>  |                           |               |             |
| 5S                               | 17                        | 15            | 14          |
| 4S                               | 82                        | 81            | 81          |

## 2. Observations on low molecular weight RNA

Where RNA is extracted and fractionated under conditions which maintain the integrity, the fractions described above are obtained (see also Kirby, 1965; Pustztai, 1966; Loening, 1967; 1968a,b,c; Strangeway, 1977).

However, the presence of hidden fractions can be revealed by specific treatments. One such treatment is to treat the rRNA to a mild heat shock at 60°C for 6-8 minutes which results in the release of a lower molecular weight fraction described as 5.8S RNA (see Lazar et al., 1969; Higo et al., 1971; Payne & Dyer, 1972; Leaver, 1973; Reijnders et al., 1973; Grierson, 1974; Berry & Berry, 1976; Hadholov & Nickolaev, 1976). This RNA is apparently complexed to the 28S fraction.

Fractionation on 2.6% gels after heat shock did not show any differences from cold treated RNA; the slight decrease in the mycelial 28S and 18S RNA of heat-treated RNA could be attributed to a slight change in loading quantity, Figure (31B). On 7.5% gels the 5.8S RNA is revealed as a new peak, Figure (31D).

When the different sporangiophore segments were examined for the fraction, it was found to be present in large quantities in unheated RNA from the tip, see Table (12). In contrast it was present in the same quantities in the other two regions only after heating, Figure (34 D,F).

Table 12

The ratio of area of  $\frac{5S}{5.85}$  of RNA from the tip, middle  
and base segments

| <u>Segments</u> | <u>Ratio</u> |
|-----------------|--------------|
| Tip             | 1.21         |
| Middle          | 1.71         |
| Base            | 1.69         |

The presence of "free" 5.8S rRNA has been observed in actively growing mycelium (Pearson, personal communication) and may represent a reflection of high activity of ribosomes or their synthesis.

4. RNA fractionation during development of sporangio-  
phores from mycelium

Low and high molecular weight species of RNA were extracted from mycelium before and after induction of sporangiophore initiation or development. Sporangiophore induction was obtained by aeration of the submerged mycelium, following the methods described in "General materials and methods".

RNA fractionation was achieved by 2.6% and 7.5% gels, and shown in Figure (35). At the initiation of sporangiophore development a new species of RNA, determined as 20S, appeared in the gel, Figure (35B). Also greater contents of 5.8S RNA were also seen in association with the initiation of the sporangiophore, Figure (35D), compared with those of the mycelium, Figure (35A,C). A new species of 20S RNA was also seen during the sporulation of Saccharomyces cerevisiae, and was considered to reflect a significant physiological state of the organism (Hopper et al., 1974; Weijksnora & Haber, 1978). 20S RNA of Phycomyces was found to disappear during subsequent growth of stage I, but appeared again at the sporulation time of sporangiophore (stage II). After spore formation was complete, 20S RNA was no longer found, thus it is possible that 5.8S and 20S RNA formation may be attributed to a unique metabolic activity during these stages of

Figure (35)

PAGE of high and low molecular weight RNA species  
extracted from mycelium, before and after sporangiophore  
development

A. 2.6% gels

1-18S rRNA

2-28S rRNA

3-DNA

B. 7.5% gels

1-4S RNA

2-5S rRNA

After sporangiophore development

C. 2.6% gel

1-18S rRNA

2-20S rRNA

3-18S rRNA

4-DNA

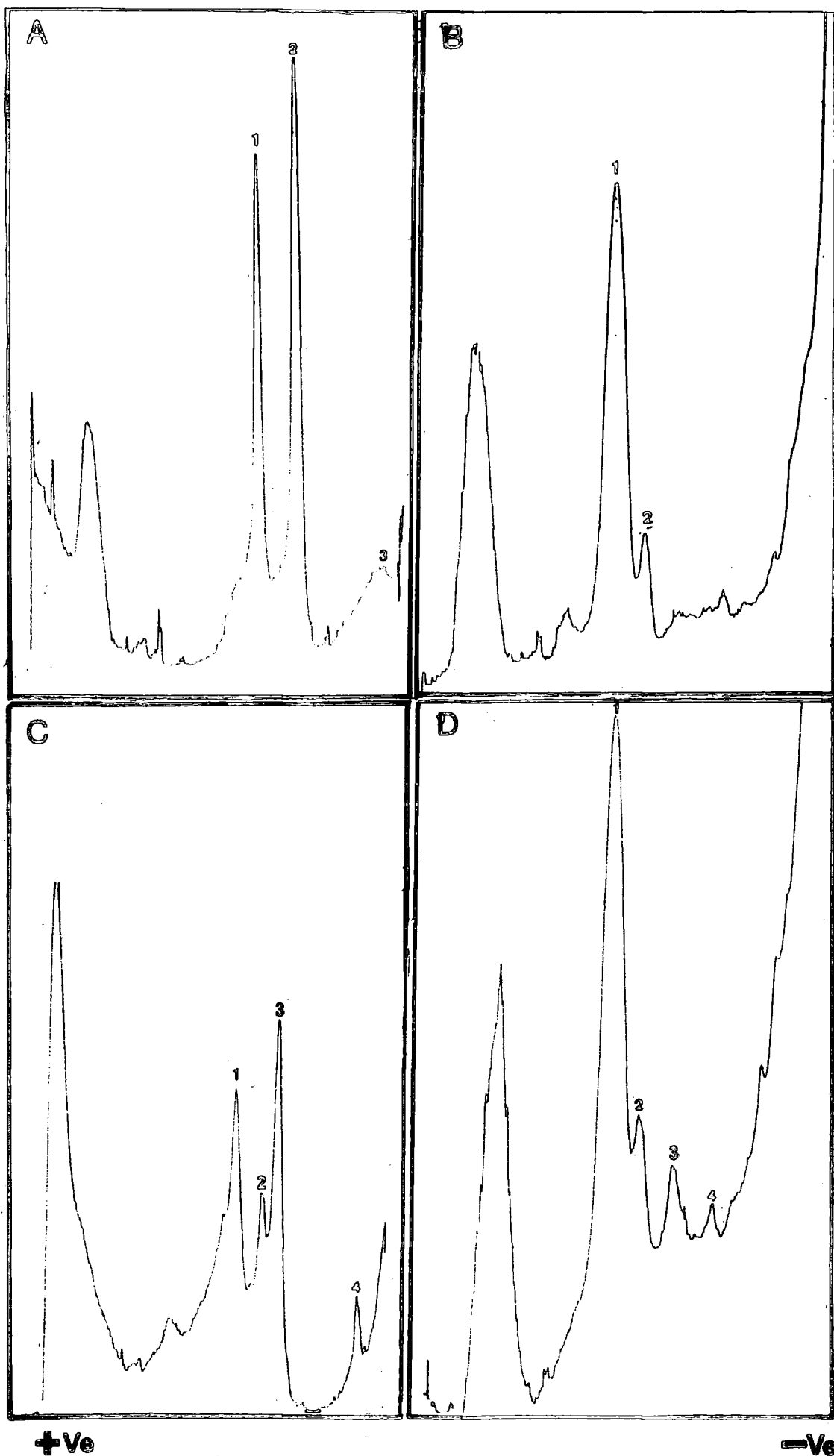
D. 7.5% gel

1-4S RNA

2-5S rRNA

3-5.8S rRNA

A<sub>265nm</sub>





differentiation and morphogenesis. Likewise a unique arrangement for synthesis of 5.8S during growth and development of yeast was also suggested by Phillipsen et al., (1978).

## 5. RNA and ageing

### 1. RNA characterization

During growth and development of sporangiophores, RNA was extracted from different regions of stage I, II plus III and IV. The absorption spectrum of total RNA showed variation in max. and min., depending on which stage of sporangiophore was examined, Figures (36, 37). The ratios of the  $\frac{E_{max}}{E_{min}}$  and  $\frac{E_{max}}{E_{280}}$  are shown in Table (13).

Changes in the absorption of RNA of stage IV could be attributed to the contamination of the RNA with other components, possibly protein or cell wall material, penetrating from the older regions.

RNA concentration also varied, and appeared to decline from stage I to stage IV. The observations appeared to be similar to the cytological and quantitative studies reported in Chapter I.

### 2. RNA fractionation

RNA species of stage I, II plus III and IV were fractionated by 2.6% and 7.5% polyacrylamide gel electrophoresis. Figures (38H, 38L, 39) show the

Figure (36)

U.v. absorption spectra of RNA extracted from

A. Stage I

B. Stage II

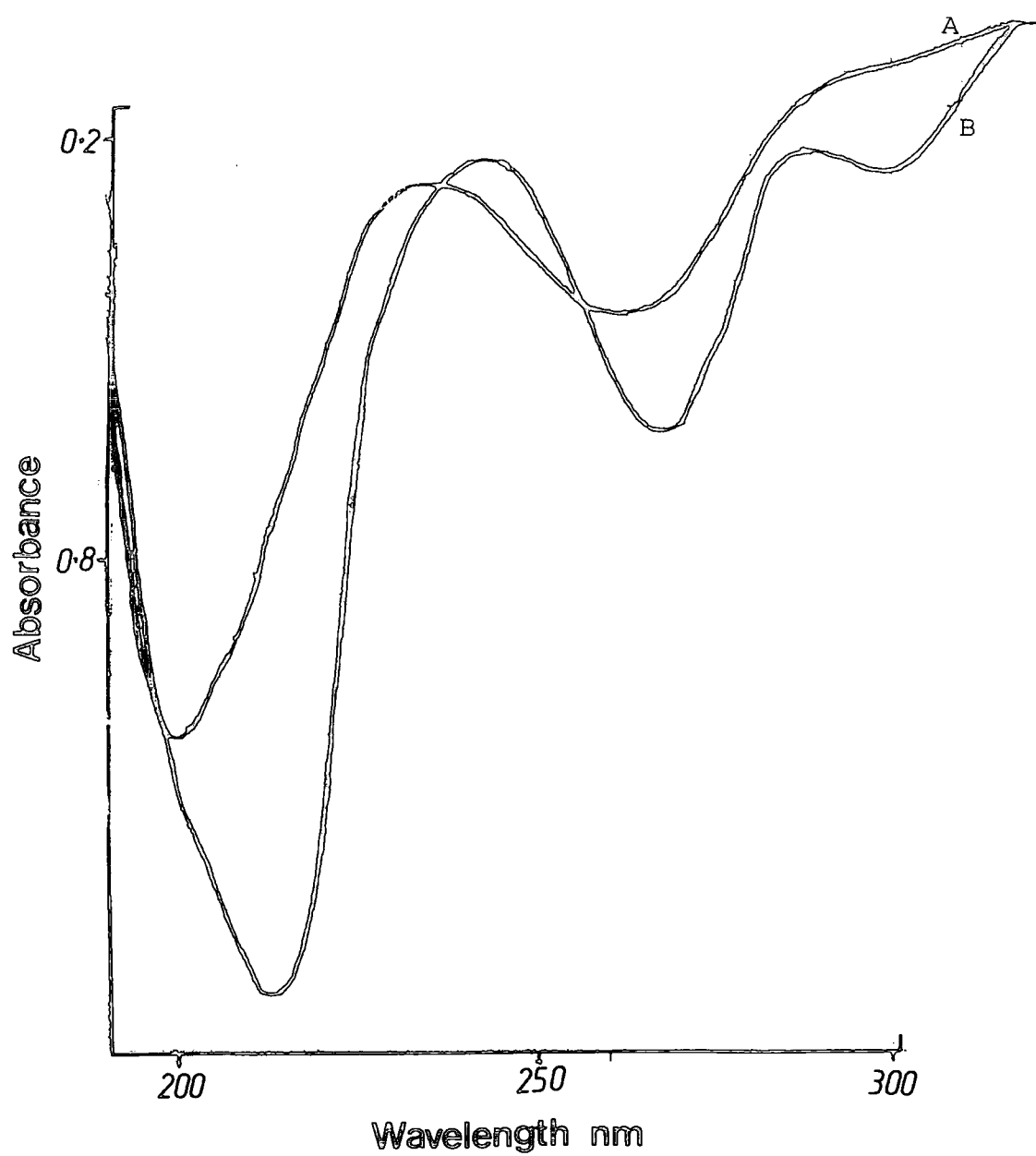


Figure (37)

U.v. absorption spectra of RNA extracted from

A. Stage I

B. Stage IV

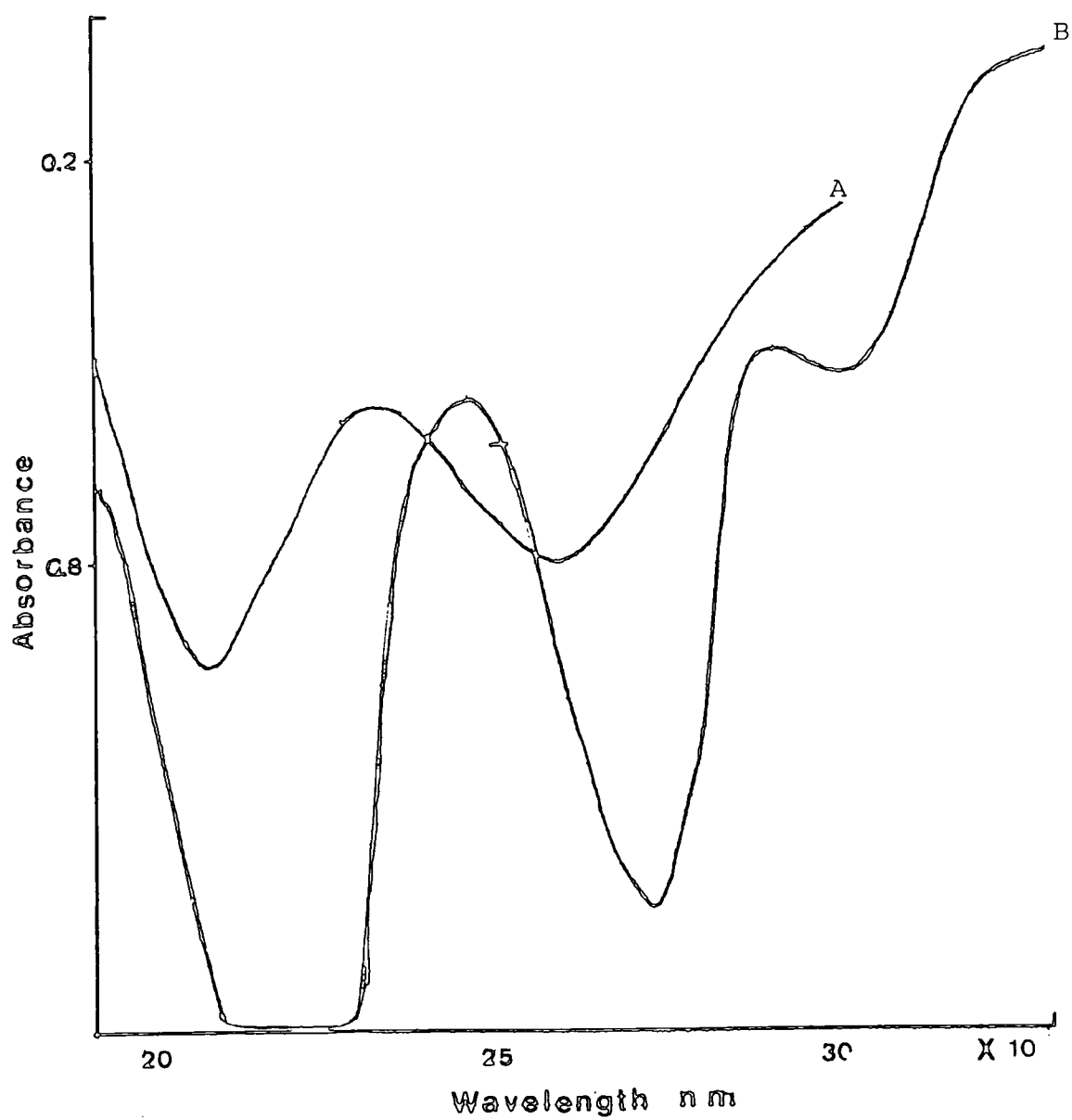


Table (13)

The ratios of  $\frac{E_{\max}}{E_{\min}}$  and  $\frac{E_{\max}}{E_{280}}$  of RNA which were extracted  
from different stages of sporangiophore

| <u>Stages</u>  | $\frac{E_{\max}}{E_{\min}}$ | $\frac{E_{\max}}{E_{280}}$ |
|----------------|-----------------------------|----------------------------|
| Stage I        | 1.58                        | 1.92                       |
| Stage II + III | 1.24                        | 1.46                       |
| Stage IV       | 1.35                        | 1.67                       |

qualitative changes of both high and low molecular weight RNA, extracted from tip and basal regions of sporangio-  
phores of stage I, II plus III and IV.

During development of sporangio-  
phores from stage I to stage II, 28S and 18S rRNA quantities were found to decrease in the basal region of the sporangio-  
phores and correspondingly increased at the tip region of the same sporangio-  
phore, see Figure (38HG,D). 28S and 18S rRNA of the basal region of stage II also showed breakdown of peaks compared with RNA species of the basal region of stage I. This may reflect RNA fragmentation. Absence of a supplement of cellular components from the substrate hyphae into the sporangio-  
phore of stage II is consistent with this (see Bergman et al., 1969), since this may lead to a decrease in the RNA of the basal region of stage II and also it may cause RNA degradation during such a condition of starvation resulting in autolysis. Alternatively an increased content of 28S and 18S rRNA at the tip region of stage II might be due to greater synthesis of RNA during the sporulation stage; such increase may also be achieved at the expense of the RNA content at the basal region.

4S and 5S RNA also showed a decrease at the basal region of stage II sporangio-  
phore, while greater quantities of 4S, 5S and 5.8S RNA were seen at the tip of stage II, Figure (38L,C,D). However, the quantities of the lower

Figure (38H)

PAGE of high molecular weight RNA species extracted from segments of different stages of sporangiophore development, fractionated by 2.6% gels

Stage I

A. Basal region

1-18S

2-28S

B. Tip region

Stage II

C. Basal region

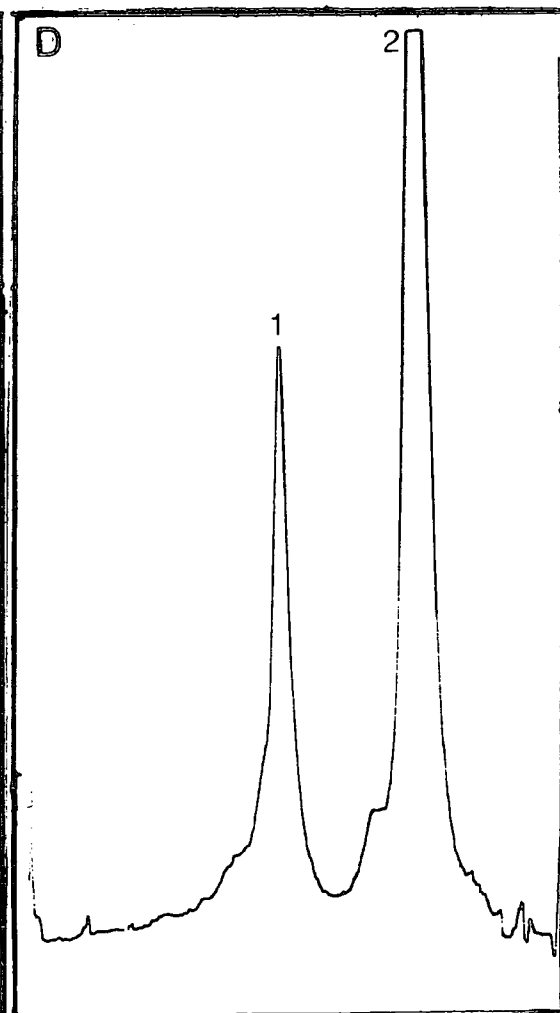
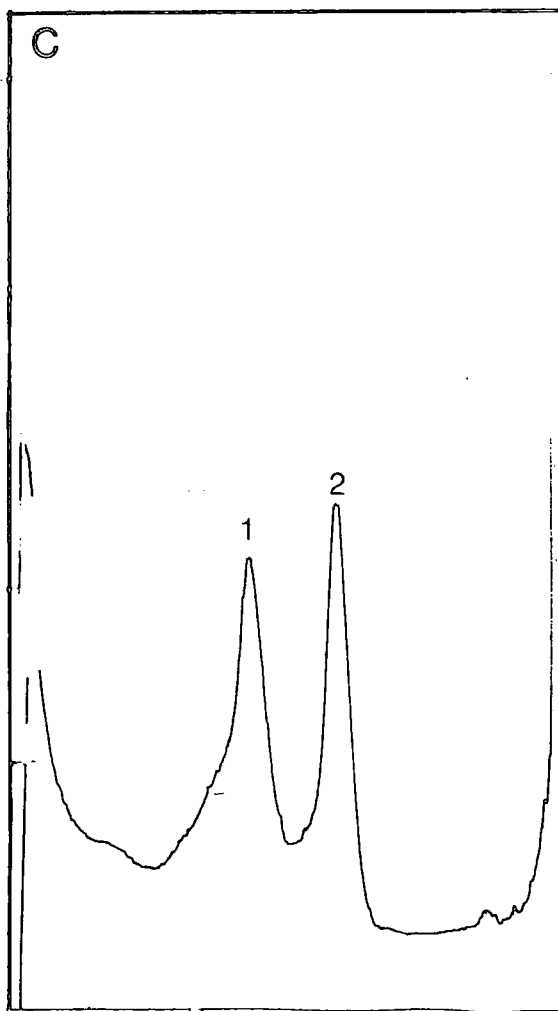
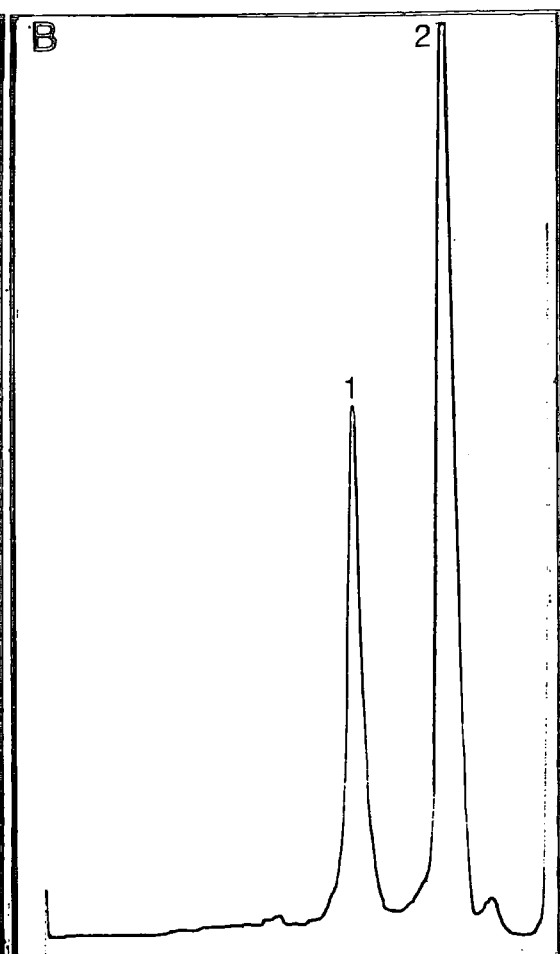
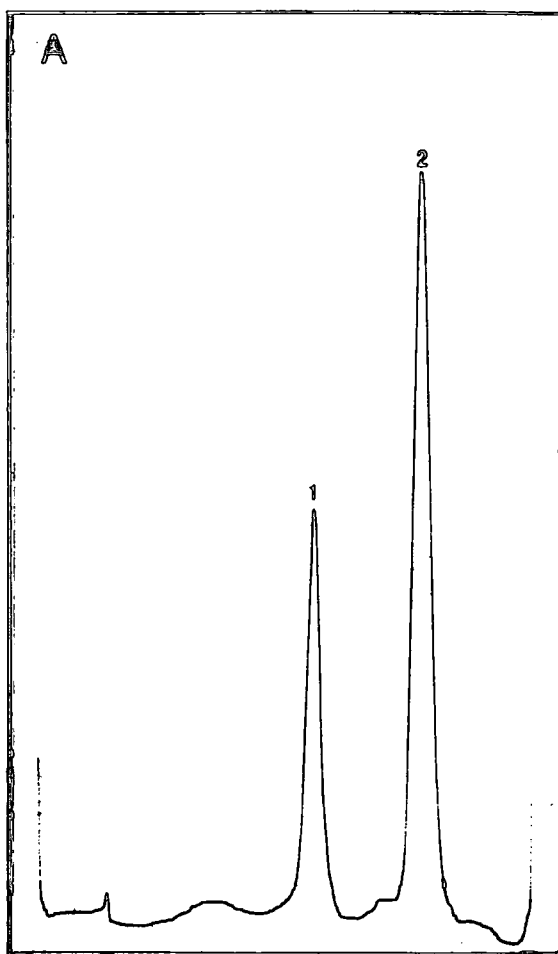
D. Tip region

1-18S

2-28S



A 265 nm



+Ve

-Ve

Figure (38L)

PAGE of low molecular RNA species extracted from different stages, and fractionated by 7.5% gels

Stage I

A. Basal region

B. Tip region

1-4S RNA

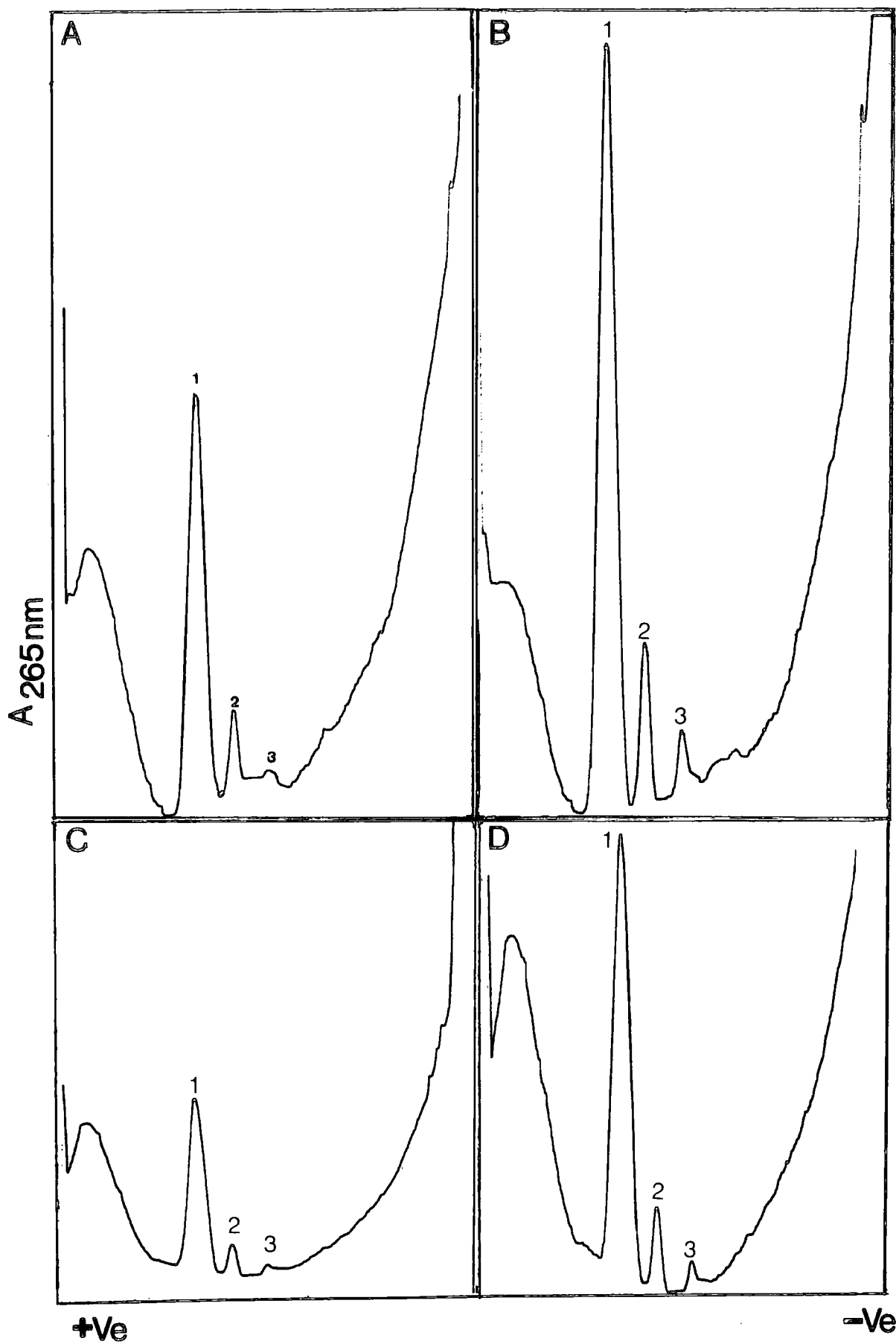
2-5S RNA

3-5.8S RNA

Stage II

C. Basal region

D. Tip region



molecular species RNA of the tip and basal regions of stage II were seen to be less than those of the low molecular species RNA of tip and basal regions of stage I respectively.

A broadening of peaks of low molecular weight were found in the low molecular species RNA extracted from the basal region of stage IV see Figure (39A) compared with the same species fractionated from the tip region of stage IV, Figure (39B). Less quantity and highly broadening of 18S rRNA species of the tip region of stage IV sporangio-  
phore was seen when compared with the same species of RNA from the tip region of the same stage, Figure (39C & D).

Degradation of RNA species at the basal region of stage IV could be attributed to the autolysis at starvation. Increased quantities of high and low molecular weight RNA species at the tip region of stage IV could be attributed to the recovery of RNA at a location of growth extension, otherwise degradation of RNA species at the basal region of stage IV might be achieved to maintain the required level of RNA precursors at the tip region of a stage IV sporangio-  
phore.

Therefore, the development of the sporangio-  
phore appeared to be associated with overall changes in the high and low molecular weight RNA species. Changes in the



Figure (39)

PAGE of low and high molecular weight RNA extracted from Stage IV

7.5% gels

A. Basal region

1-4S RNA

2-5S RNA

3-5.8S RNA

B. Tip region

1-4S RNA

2-5S RNA

3-5.8S RNA

2.6% gels

C. Basal region

1-18S rRNA

2-28S rRNA

3-DNA

D. Tip region

1-18S rRNA

2-28S rRNA

3-DNA

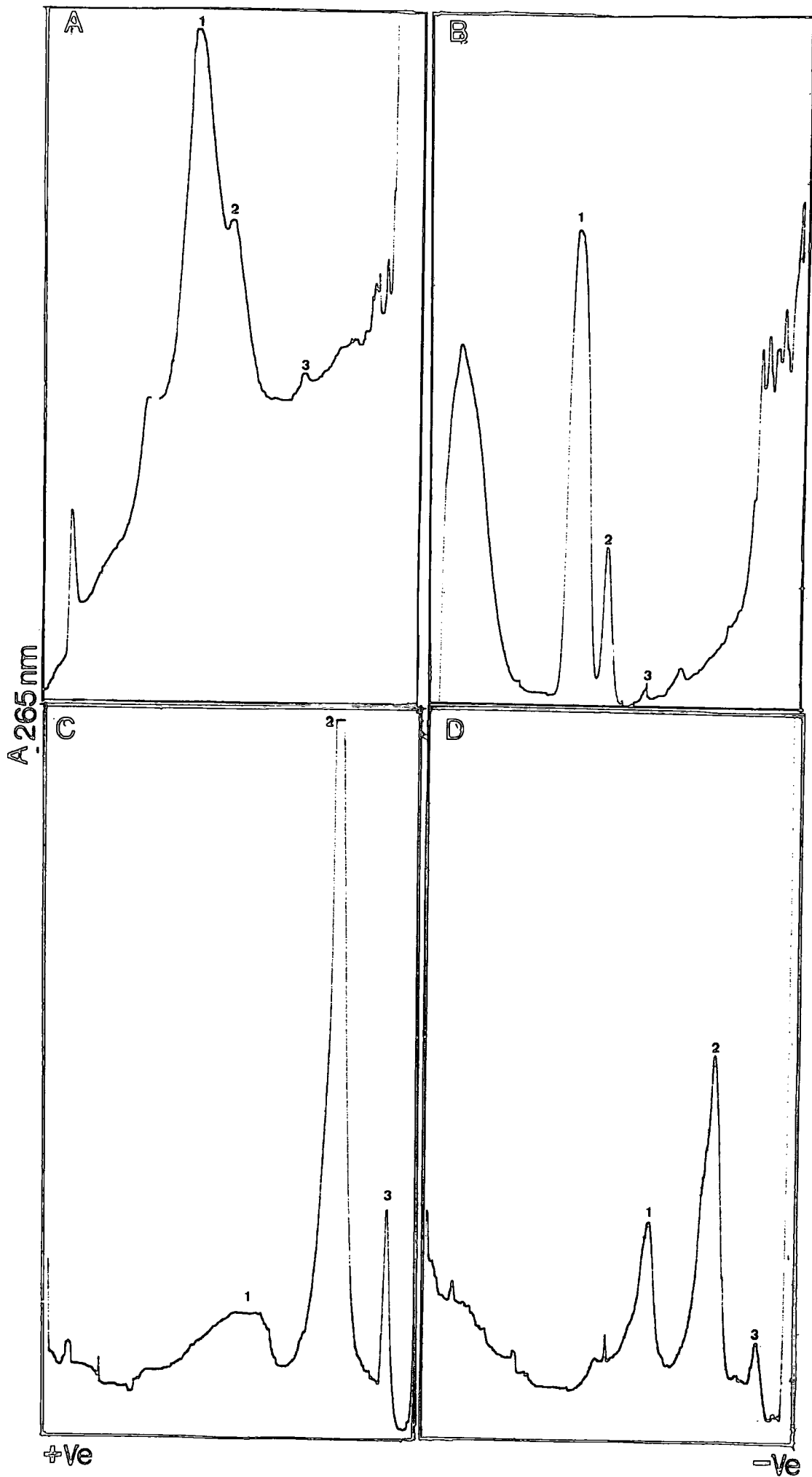


Figure (40)

PAGE of high molecular weight RNA species extracted from tip, middle and basal segments during regeneration, 2.6% gels were used

Tip

A. At 2 hours

B. At 8 hours

1-18S rRNA

2-28S rRNA

Middle

C. At 2 hours

D. At 8 hours

Base

E. At 2 hours

F. At 8 hours



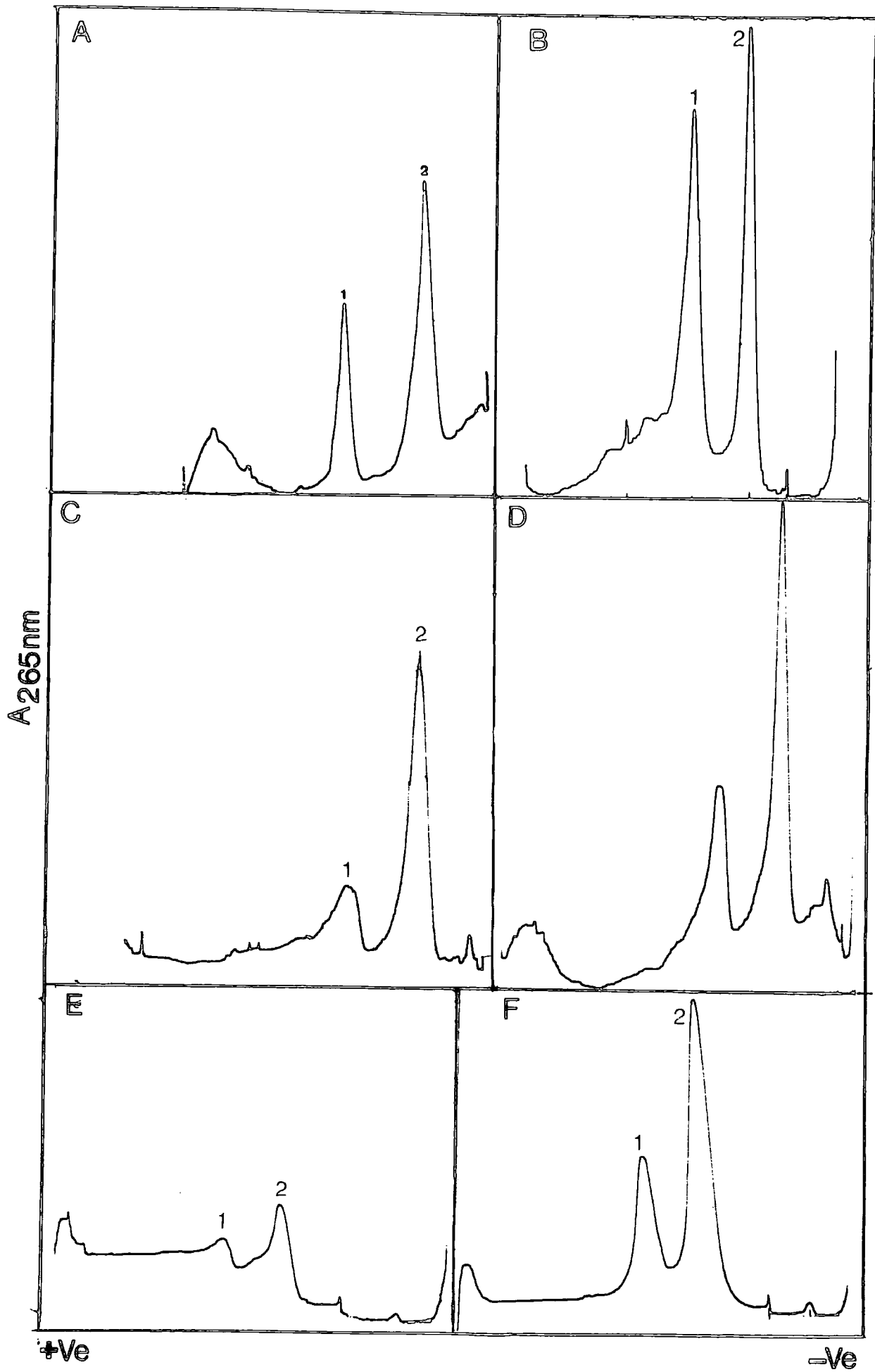


Figure (41)

PAGE of low molecular weight RNA species extracted from tip, middle and basal segments during regeneration. 7.5% gels were used

Tip

A. At 2 hours

B. At 8 hours

1-4S RNA

2-5S rRNA

3-5.8S rRNA

Middle

C. At 2 hours

D. At 8 hours

1-4S RNA

2-5S RNA

3-5.8S rRNA

Base

E. At 2 hours

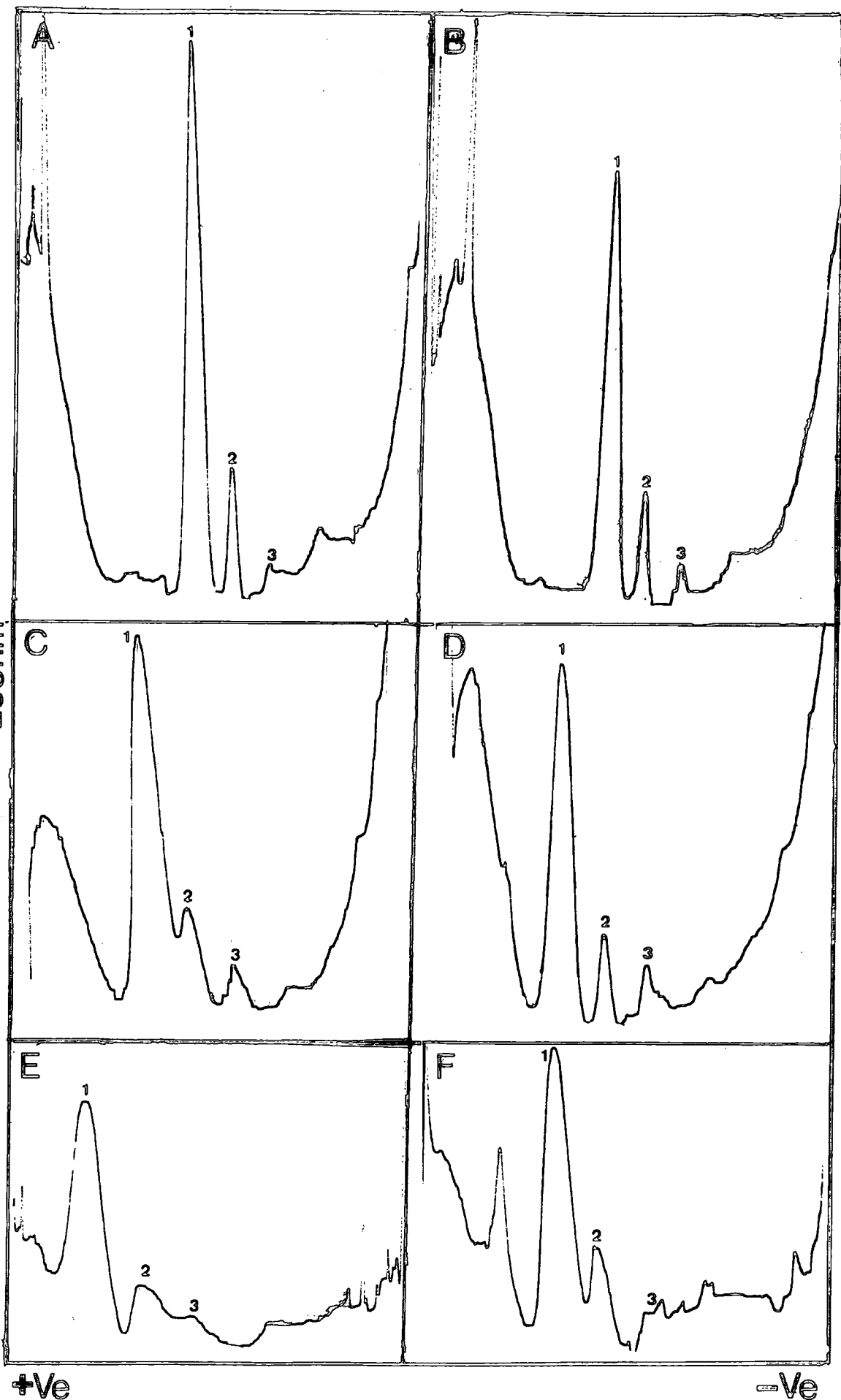
F. At 8 hours

1-4S RNA

2-5S rRNA

3-5.8S rRNA

A 265nm



+Ve

-Ve

quantitative fraction (Chapter I) can be attributed here to the decrease in the quantities of specific RNA species which may be caused by degradation or starvation-induced autolysis.

6. RNA fractionation during the regeneration of sporangiophores

RNA was extracted from tip, middle and basal segments during regeneration. The extraction was carried out at different time intervals. The segments were equal in length and cultured on solid medium. RNA extracted from segments at zero time (0h), after cutting was used as a control.

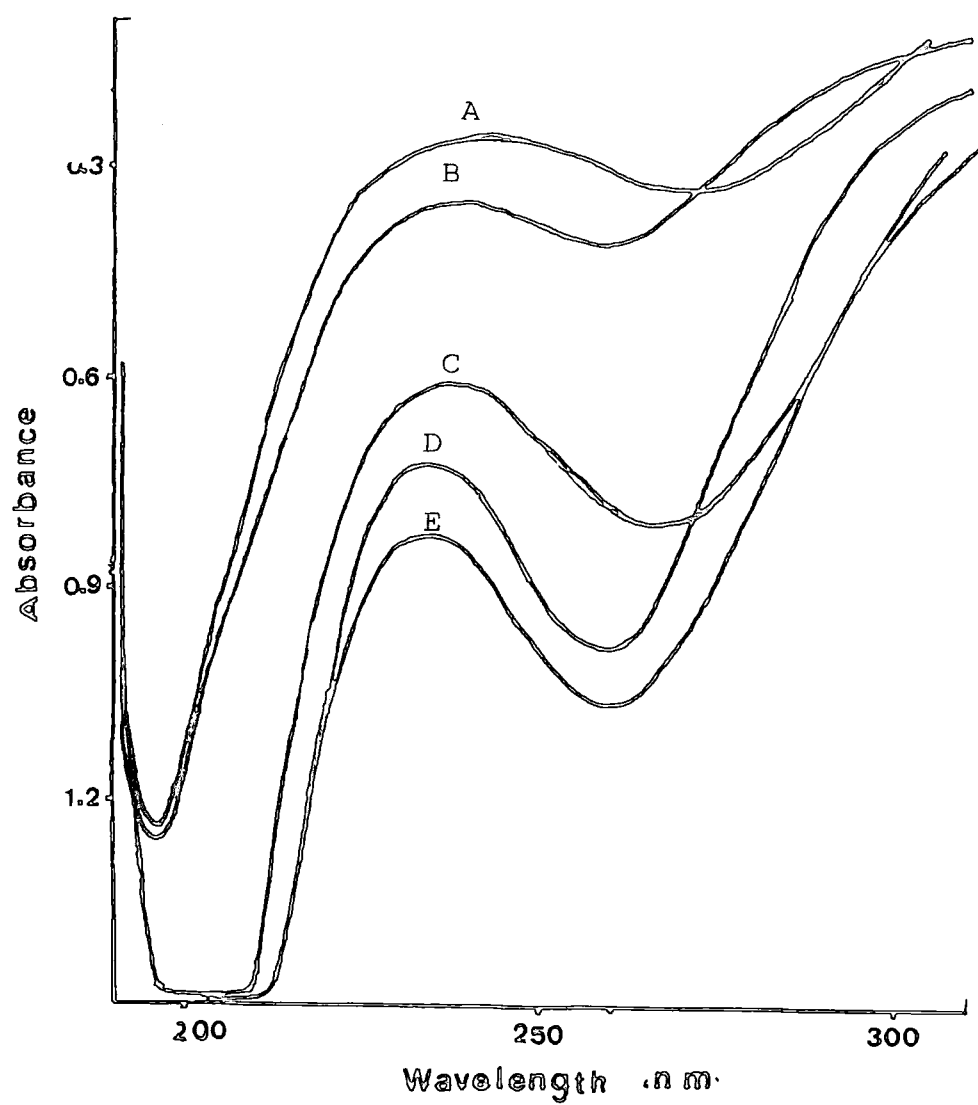
RNA was also fractionated on 2.6% and 7.5% gels and using a similar system for electrophoresis as described above. Figures (40,41) show only the fractionations of RNA extracted from segments before and after regeneration.

After 2 hours of manipulation, there was a broadening of the peaks of high and low molecular weight RNA species, of all segments as shown in Figure (40A,C,E) and in Figure (41A,C,E). No such broadening was seen in the fraction of RNA species of the control. Broadening of the peaks is suggested to be due to breakdown of the RNA. Such breakdown decreased from the base to the tip segment fractions and this suggested it may be attributed to the

Figure (42)

U.v. absorption spectra of RNA extracted during  
regeneration segments

- A. Basal segments
- B. Middle segments                      after 2 hours
- C. Tip segments
- D. Tip segments after 8 hours
- E. Full length sporangiophore at 0 hours



effect of mechanical shock. During this period of culturing, the quantities of RNA showed a decrease, and this is in agreement with the observations found in Chapter I where the initial decrease of RNA could be attributed to breakdown of RNA species.

U.v. absorption spectra also showed changes in the absorbance compared with the control, Figure (42). A suitable explanation for this is a possible release of protein during cutting, or due to a contamination of the extractant with cell wall materials.

At 8 hours of culturing, tip segments were shown to develop sporangiophores at the cut site. At the same time, high molecular weight RNA species showed less broadening compared with the 2 hours fraction. RNA fractions of middle and basal segments also showed a recovery in RNA level, where less broadening was seen. At 8 hours no development of sporangiophores was seen on the middle and basal segments. A maximal recovery of RNA level was achieved at 12 and 14 hours after culturing of middle and basal segments respectively, and found to be associated with the development of sporangiophores, see Figures (40 (B,D,F), 41(B,D.F)).

Such observations are in agreement with cytological and quantitative studies reported in Chapter I. The

breakdown of the RNA also can be compared with internal damage which was found after the cutting, by the staining method, at the area under stress. The absence of breakdown in the RNA profile of the control revealed that the immediate freezing of the tissue in liquid nitrogen may prevent the effect.

The accumulation of RNA at the site of regeneration, appeared here to be associated with the recovery of RNA species. The recovery showed a variation from segment to segment. The tip region showed a quicker recovery in the level of RNA species than the middle and base segments. This could be due to the presence of more cellular components at the tip region compared with the others.

7. Effect of RNA and protein synthesis inhibitors on the fractionated RNA during growth of isolated sporangiophores

The effect of 6-methylpurine ( $25 \mu\text{g}.\text{ml}^{-1}$ ), actinomycin D ( $25 \mu\text{g}.\text{ml}^{-1}$ ), rifampicin ( $25 \mu\text{g}.\text{ml}^{-1}$ ) and cycloheximide ( $20 \mu\text{g}.\text{ml}^{-1}$ ) on the recovery of RNA species during the regeneration of sporangiophores was tested. Methods used for isolating and manipulation were similar to those described in Chapter I.

RNA was extracted at different intervals during the treatments, and fractionated by 2.6% and 7.5% gels system.



Fractionation of RNA extracted from sporangiophores cultured in water were used as a control. Figures (43, 44, 45, 46) show high and low molecular fractionation of RNA extracted at 6 and 24 hours after culturing.

At 6 hours after the time of commencement there was no inhibition of growth of sporangiophores, by any of the inhibitors. Additionally there were no differences in the RNA species of both high and low molecular weight compared with the control as shown in Figures (43(A,B), 44(A,B)).

After 6 hours, the growth of sporangiophores cultured in 6-methylpurine and cycloheximide was inhibited. This was shown to be associated with the release of fragments which appeared as a shoulder on high molecular weight rRNA species as shown in Figure (43(D,E)). Such fragmentation could be due to the breakdown of RNA species as a result of inhibitor action. No fragments were seen in the 7.5% gel fractions, but broadening of 4S and 5S peaks and a lower level of 5.8S RNA were observed compared with control Figures (44D, 45B). After 6 hours, there were no differences in the fractions of RNA extracted from sporangiophores treated with rifampicin and actinomycin D, where growth continued as for the control.

No inhibition of growth of sporangiophores treated with rifampicin and actinomycin D was seen and they

Figure (43)

PAGE of high molecular weight RNA species extracted during growth of isolated sporangiophores treated with inhibitors

Control (in water)

A. At 6 hours

B. At 10 hours

1-18S      2-28S

Treated with 6-methylpurine ( $25 \mu\text{g}.\text{ml}^{-1}$ )

C. At 6 hours

D. At 10 hours

Treated with rifampicin ( $25 \mu\text{g}.\text{ml}^{-1}$ )

E. At 6 hours

F. At 10 hours

Treated with cycloheximide ( $20 \mu\text{g}.\text{ml}^{-1}$ )

G. At 6 hours

H. At 10 hours

1 & 3 Fragment from 18S and 28S rRNA

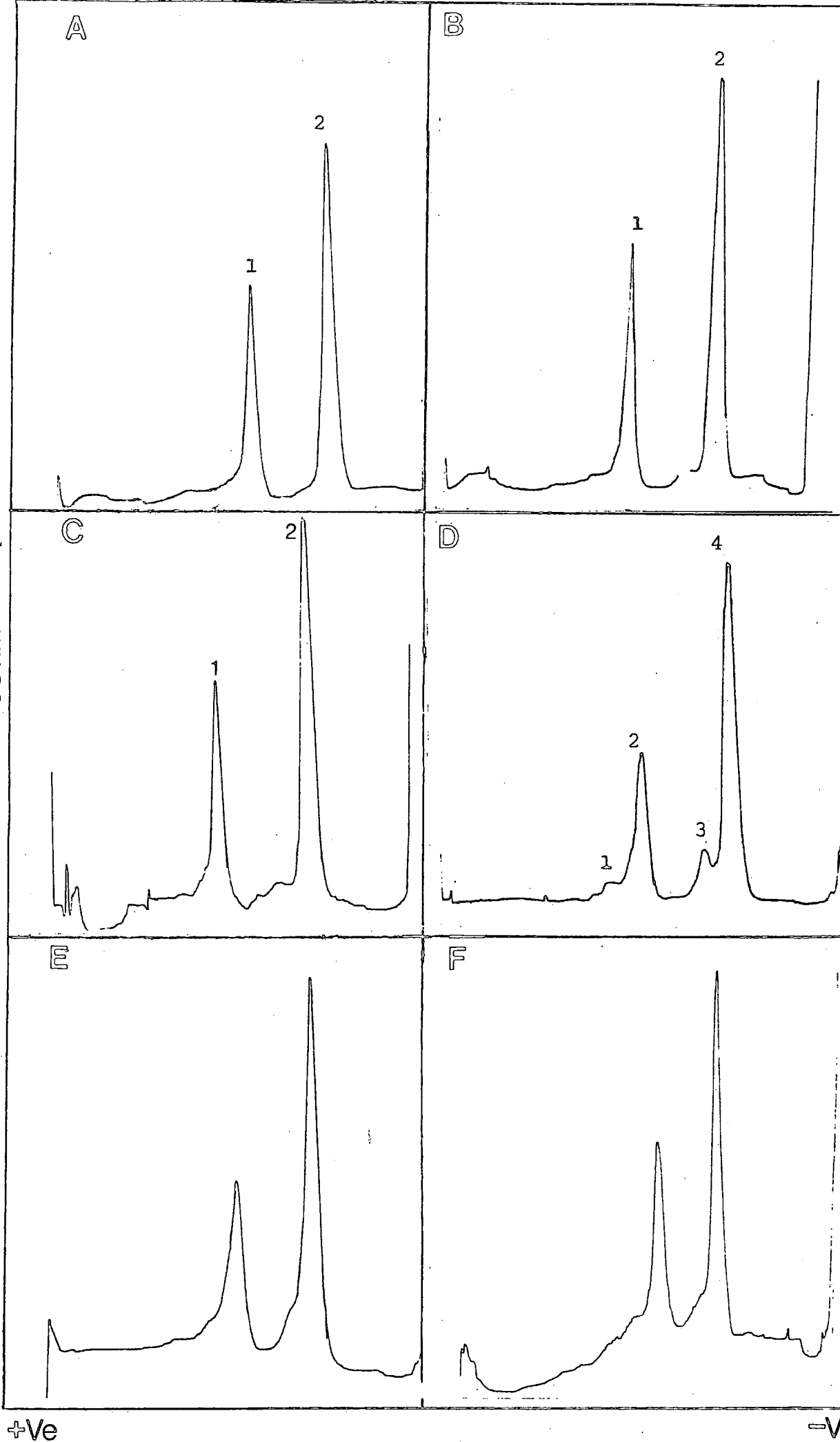
2 & 4 Normal fraction of 18S and 28S rRNA

Treated with actinomycin D ( $25 \mu\text{g}.\text{ml}^{-1}$ )

I. At 6 hours

J. At 10 hours

A 265nm



+Ve

-V

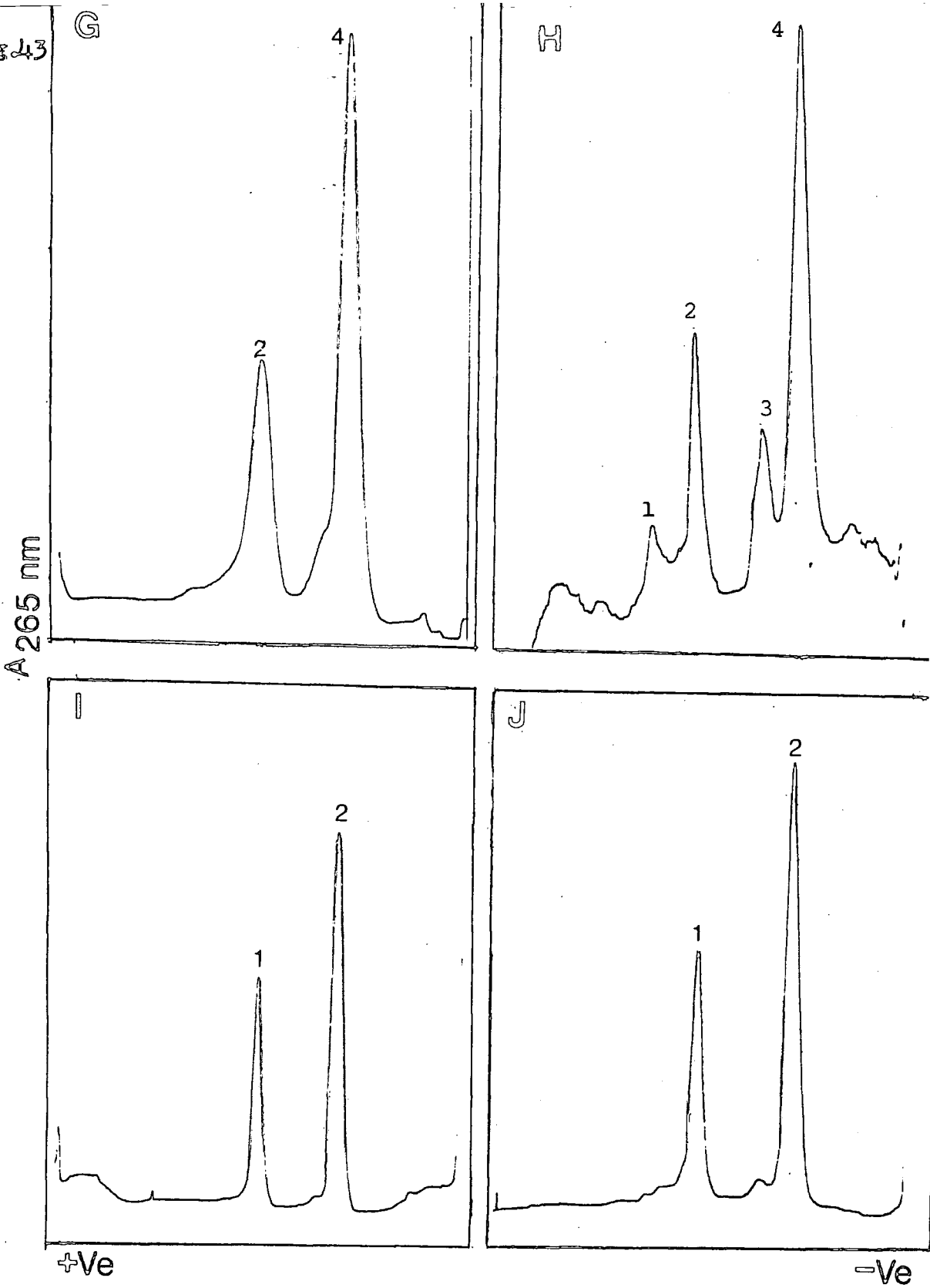


Figure (44)

PAGE of low molecular weight RNA species extracted from isolated sporangiophores treated with inhibitors

Control (in water)

A. At 6 hours

B. At 10 hours

Treated with 6-methylpurine ( $25 \mu\text{g}.\text{ml}^{-1}$ )

B. At 6 hours

D. At 10 hours

Treated with rifampicin ( $25 \mu\text{g}.\text{ml}^{-1}$ )

E. At 6 hours

F. At 10 hours

Treated with cycloheximide ( $20 \mu\text{g}.\text{ml}^{-1}$ )

G. At 6 hours

H. At 10 hours

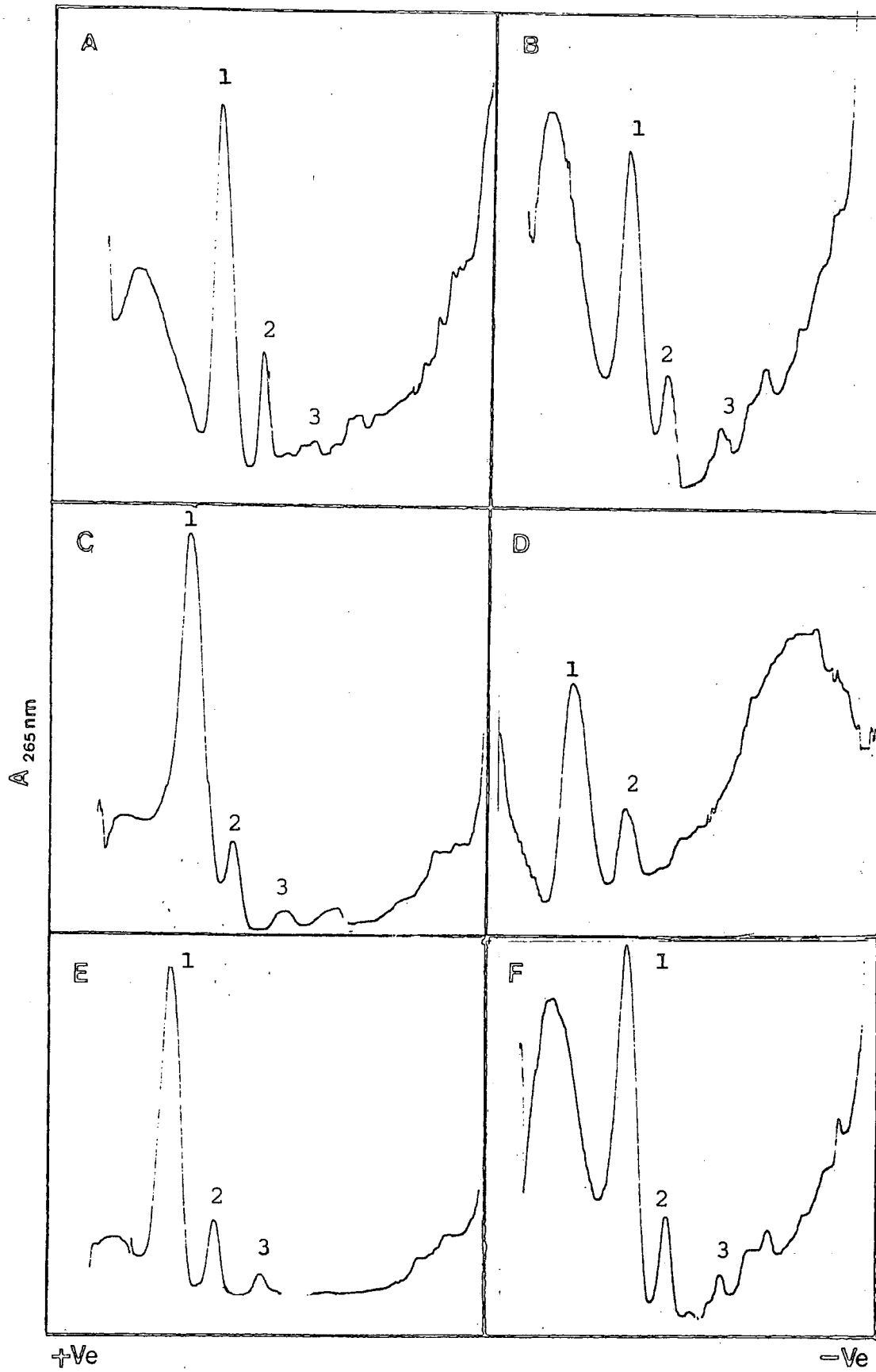
Treated with actinomycin D ( $25 \mu\text{g}.\text{ml}^{-1}$ )

I. At 6 hours

J. At 10 hours

- 1 - 4S RNA
- 2 - 5S RNA
- 3 - 5.8S RNA

D



+Ve

-Ve

Figure (45)

PAGE of low molecular weight RNA species extracted from isolated sporangiophores treated with inhibitors

Treated with cycloheximide ( $20 \mu\text{g}.\text{ml}^{-1}$ )

A. At 6 hours

B. At 10 hours

Treated with actinomycin D ( $25 \mu\text{g}.\text{ml}^{-1}$ )

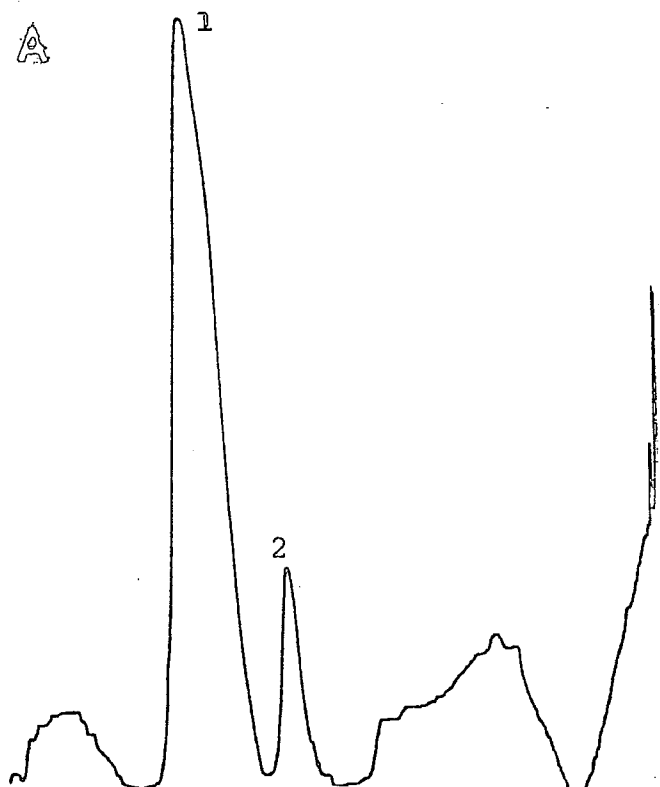
C. At 6 hours

D. At 10 hours

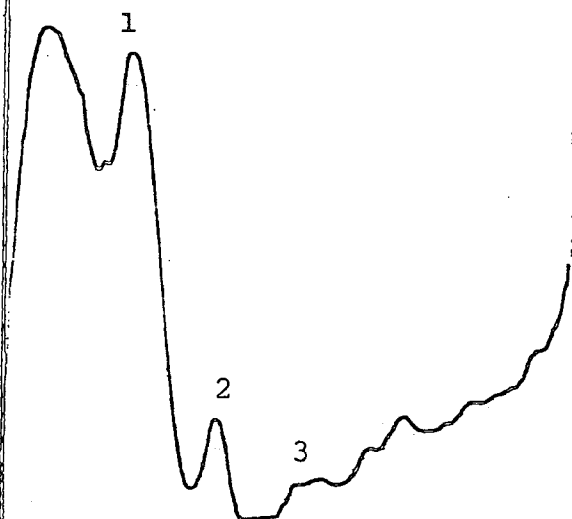
1-4S RNA  
2-5S RNA  
3-5.8SRNA

A 265nm

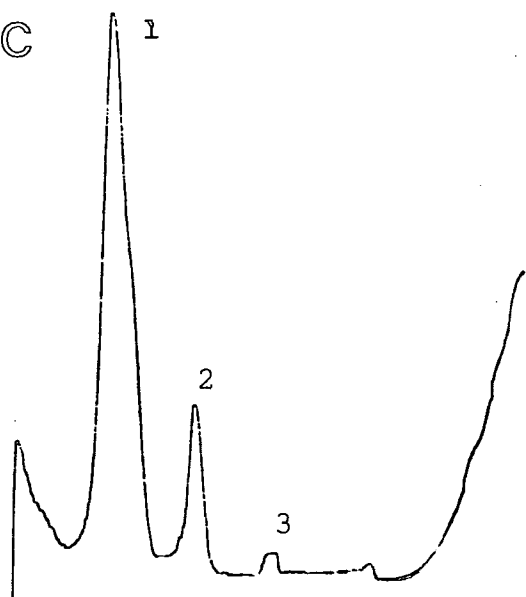
A



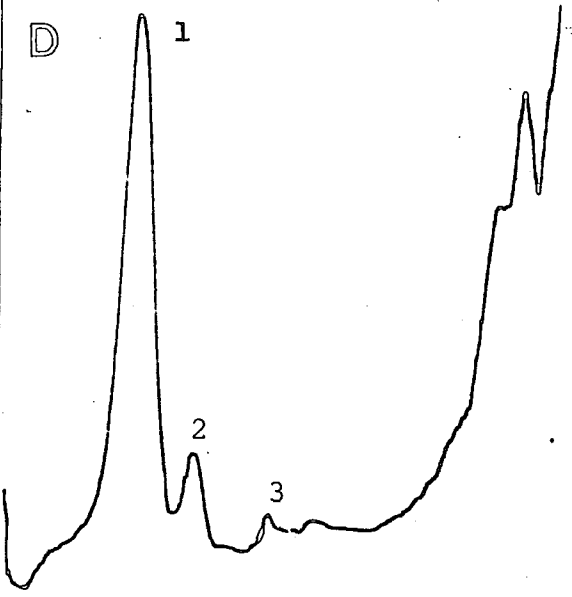
B



C



D



+Ve

-Ve



Figure (46)

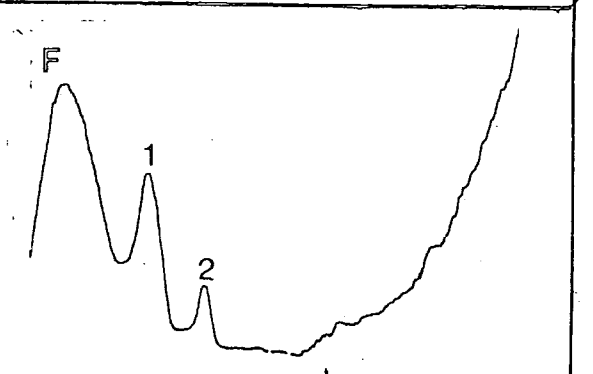
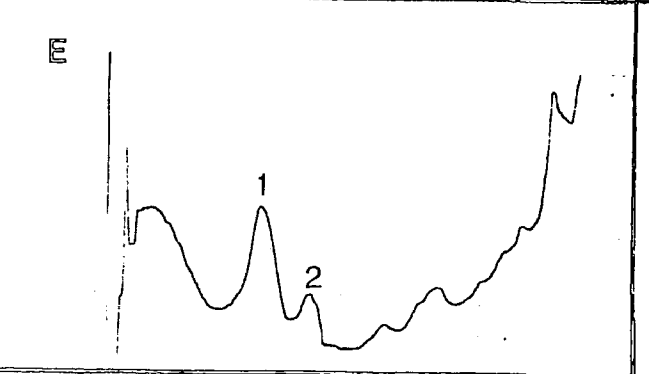
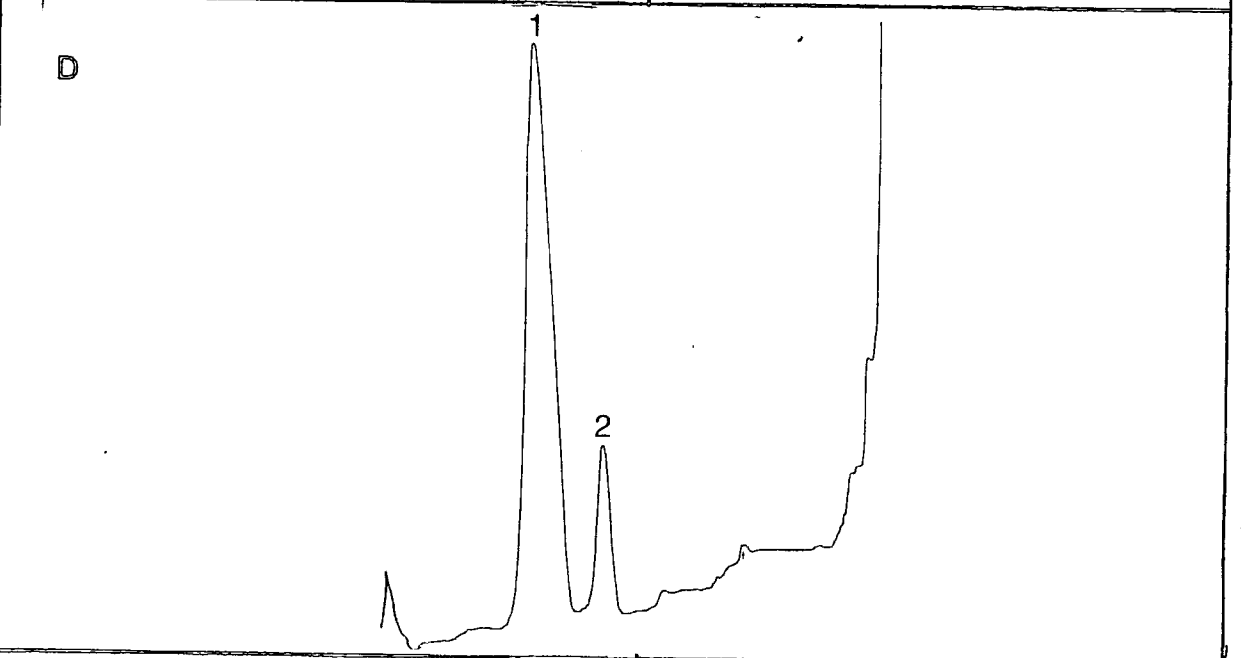
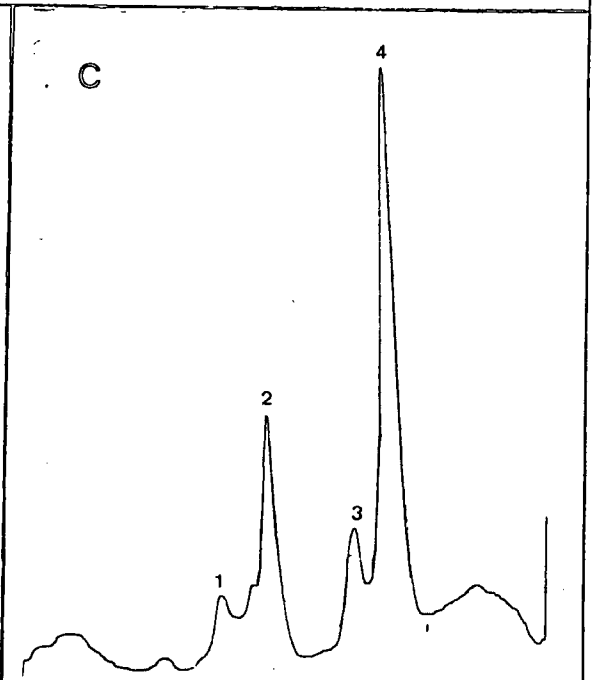
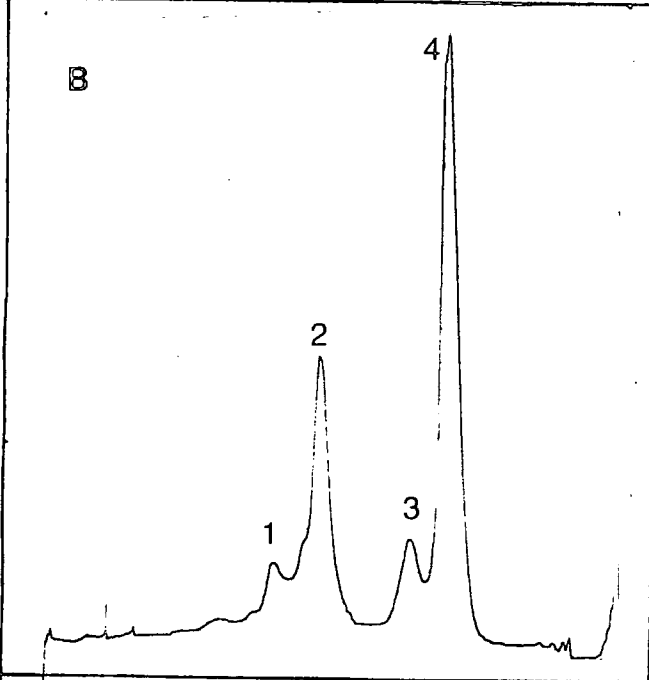
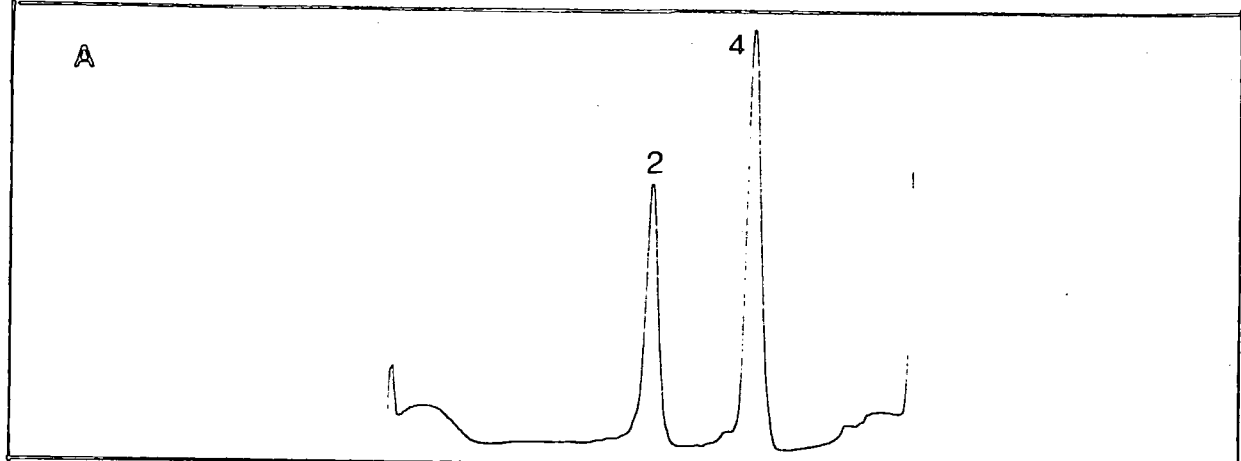
PAGE of high and low molecular weight RNA species extracted from Stage IV sporangiophores which developed in actinomycin D and rifampicin. RNA extracted from sporangiophores cultured in water was used as a control

2.6% gels

- A. High molecular weight rRNA of control
- B. High molecular weight rRNA of sporangiophores treated with rifampicin ( $25 \mu\text{g}.\text{ml}^{-1}$ ) showing breakdown
- C. High molecular weight rRNA of sporangiophores treated with actinomycin D ( $25 \mu\text{g}.\text{ml}^{-1}$ ) showing breakdown
  - 1. Fragment of 18S rRNA
  - 2. 18S rRNA
  - 3. Fragment of 28S rRNA
  - 4. 28S rRNA

7.5% gels

- D. Low molecular weight RNA species of control
- E. Low molecular weight RNA species of sporangiophores treated with rifampicin ( $25 \mu\text{g}.\text{ml}^{-1}$ ) showing less level and broadening of RNA peaks
- F. Low molecular weight RNA species of sporangiophores treated with actinomycin D ( $25 \mu\text{g}.\text{ml}^{-1}$ ) showing less level and broadening of RNA peaks
  - 1. 4S RNA
  - 2. 5S RNA



A 265nm

reached a final stage of development (IV), but the final length was less than that achieved by sporangiophores grown in water. Such observations are also in agreement with the results reported in Chapter I. At stage IV development (24 h), the RNA was extracted from sporangiophores which were treated with rifampicin, actinomycin D, and from those used as a control and Figure (46) shows the fractionation of high and low molecular weight RNA species. As shown there was a breakdown in the RNA species by release of fragments in 2.6% gels and broadening of peaks in 7.5% gels Figure (46B (C,E,F)). Such breakdown of RNA after stage IV development could be correlated with the decrease in the final length of sporangiophore since no such breakdown was seen in the RNA of control, Figure (46(A,D)).

However, cycloheximide and 6-methylpurine inhibited the growth of stage I sporangiophores, and this was reflected in the breakdown and impaired recovery of RNA. The increase in the level of high molecular weight RNA species, 28S and 18S of sporangiophores which were treated with cycloheximide could be attributed to an increase in the concentration of RNA synthesized but not utilized for protein synthesis (see <sup>Warner et al., 1966;</sup> Hadholov & Nickolaev, 1976). The inhibition of biosynthesis of

5.8S RNA by cycloheximide may reflect a functional activity of cycloheximide inhibitor in either preventing the release of 5.8S rRNA or by blocking steps in its maturation. Since it has been reported that the initiation effect of spore germination of Phycomyces by cycloheximide could be attributed to its effect on the processes of protein synthesis at the translational level and thus may inhibit some enzymes involved in cell wall synthesis (Van Laere et al., 1976, 1977), here the inhibition of 5.8S RNA could be affecting the wall synthesis at the translational level. Likewise the failure of actinomycin D to inhibit the growth could be attributed to the fact that the sporangiophore growth dependent on preformed m-RNA at the translational level, and not on the new synthesis of mRNA, since actinomycin D is a potent inhibitor of m-RNA synthesis (see Kersten & Kersten, 1974).

## 27. RESULTS OF RIBOSOME EXTRACTION AND FRACTIONATION

### 1. Characterization of the method

The u.v. absorption spectrum of ribosomes extracted from stage I sporangiophores, 15-20 mm in length, and of 1 gm fresh weight, is shown in Figure (47). The maximum and minimum absorption were at 260 and 238 nm respectively. The ratio of  $\frac{260}{238}$  and  $\frac{260}{280}$  were given as values of 1.51 and 1.76, which are in agreement with values obtained in testing the purity of ribosomes (see Kliffen, 1970; Strangeway, 1977). The average yield of the ribosome was of the order of 1400-1600  $\mu$ g ribosomes per gm tissue. Not much difference was found comparing these ratios with that of mycelium (Pearson & Thomas unpublished work and Strangeway, 1977).

### 2. Polyacrylamide gel electrophoresis fractionation

Sporangiophore ribosomes were electrophoresed and fractionated by 2.2% polyacrylamide gel electrophoresis system (see materials and methods). Fractionation profiles of ribosomes are shown in Figure (48(B,D)). Up to 8 peaks of the ribosomal polymer fractionation were observed. Those peaks were nomenclatured as monomer; dimer, trimer, tetramer, pentamer, hexamer, septamer and octamer. These peaks represent increased numbers of ribosomes attached to the mRNA, in which the fractions near the top

Figure (47)

U.v. absorption spectrum of ribosomes extracted from  
sporangiophores

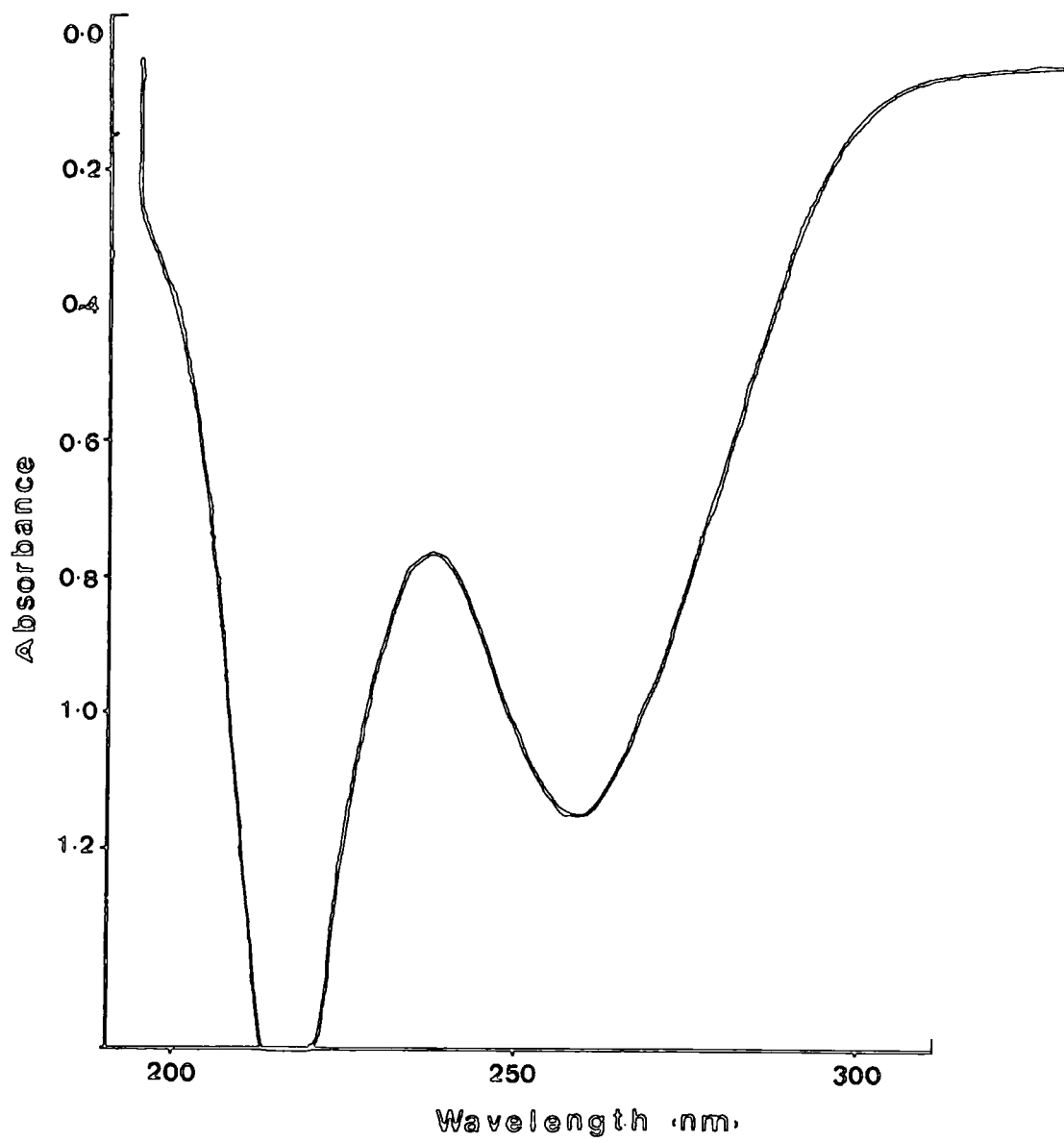


Figure (48)

PAGE of polyribosomes extracted from stage I sporangiophores and fractionated on 2.2% gels

A & C. Polyribosomes were treated with  $10 \text{ mg.ml}^{-1}$  RNAase before fractionation

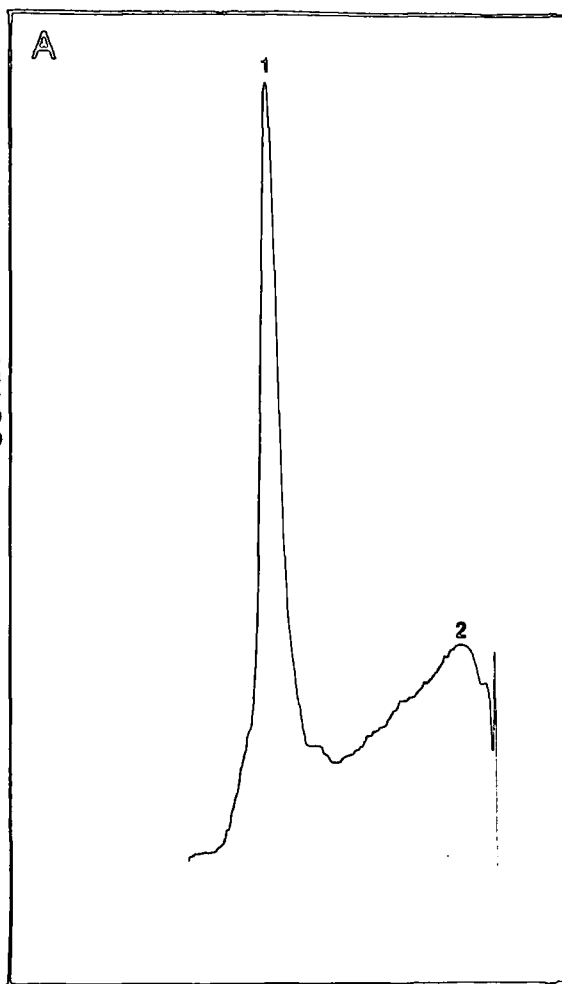
1. Monosomes
2. protein

C & D. Non-treated

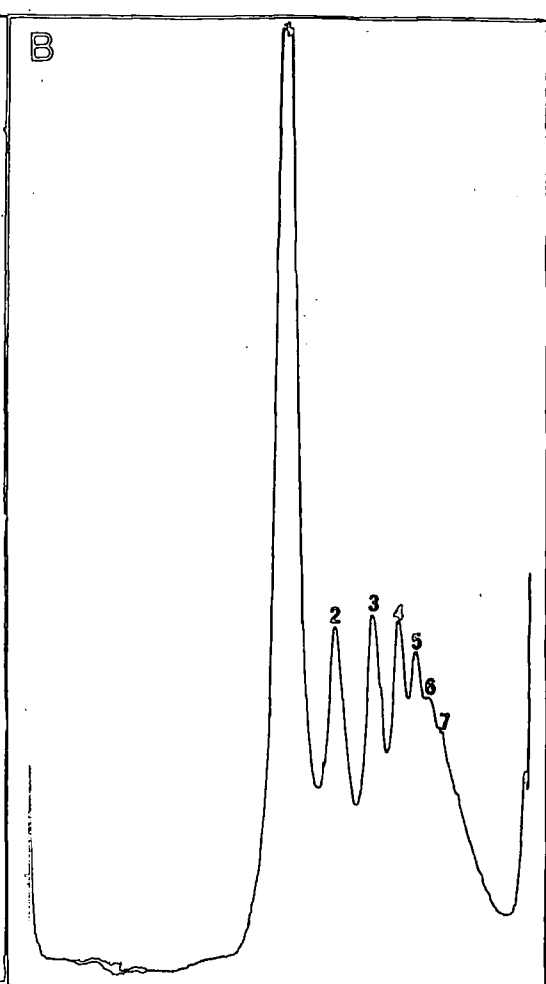
1. Monomer
2. Dimer
3. Trimer
4. Tetramer
5. Pentamer
6. Hexamer
7. Septamer
8. Octamer



A 265 nm

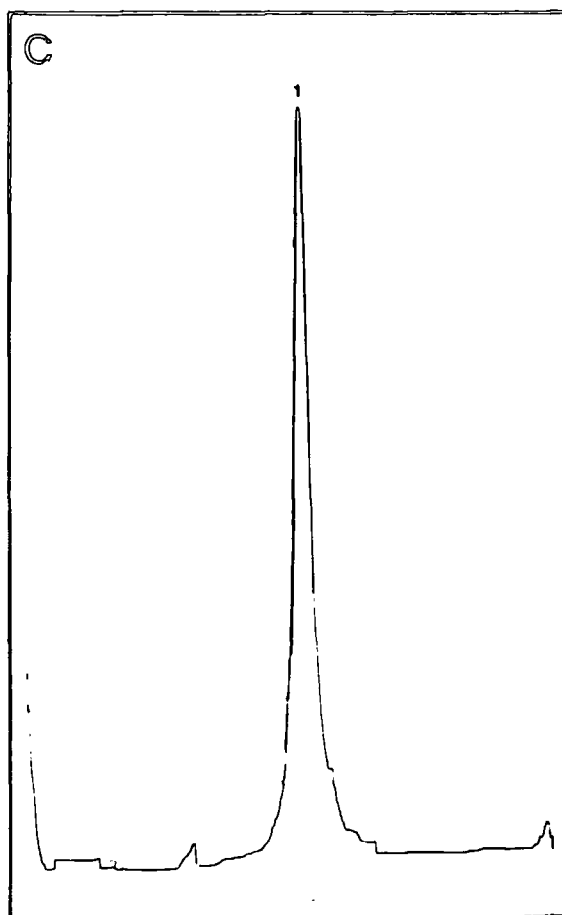


+Ve

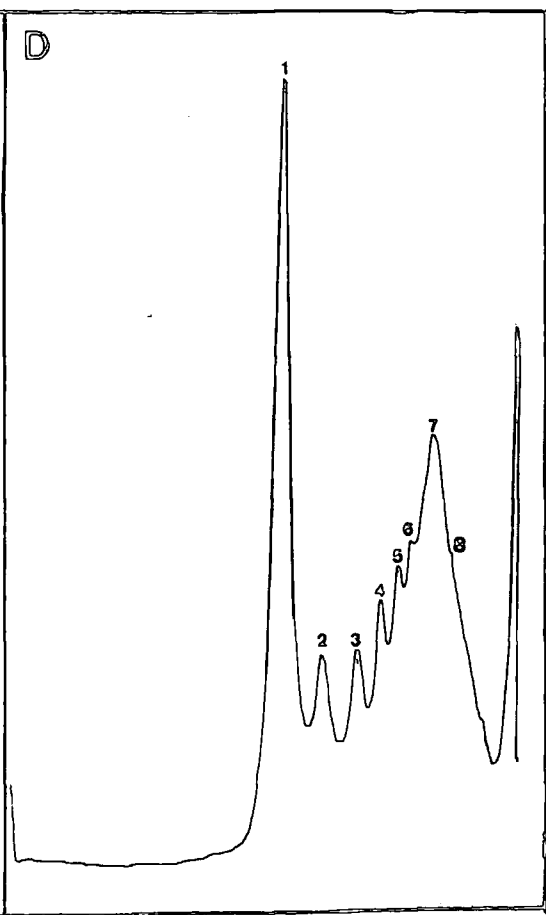


-Ve

A 265 nm



+Ve



-Ve

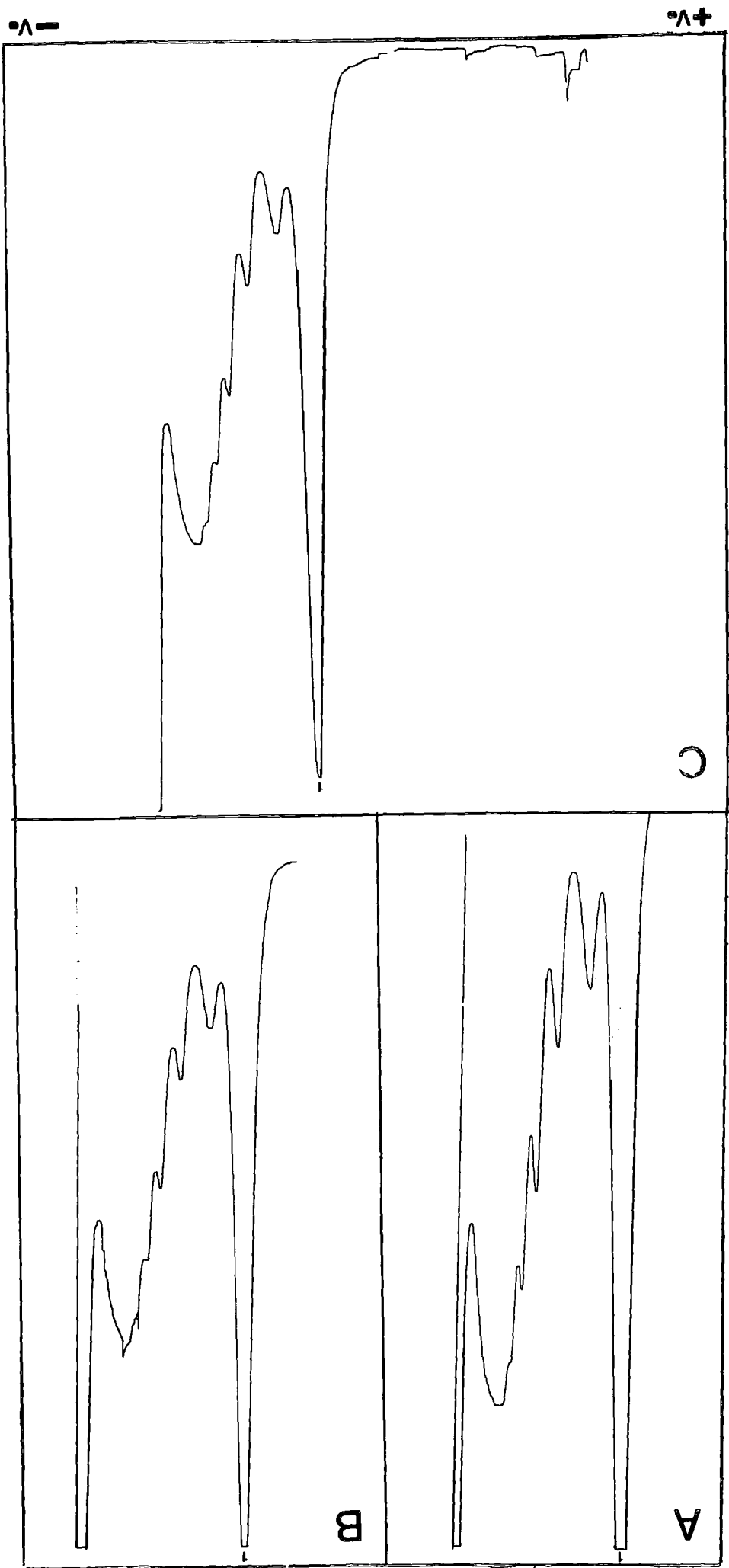
Figure (49)

PAGE of polyribosomes extracted from stage I sporangiophores  
fractionated on 2.2% and loaded with different concentrations

- A. 15  $\mu$ g ribosomes
- B. 20  $\mu$ g ribosomes
- C. 25  $\mu$ g ribosomes

1 - Monosomes

A 265nm



of the gel can be considered as high polymers (polysomes) aggregation (Strangeway, 1977).

15µg ribosome was found to be a suitable loading on 2.2% gel Figure (49C), since greater amount of loading (20 µg and 25 µg ribosomes) showed a bad resolution and separation of ribosomes, see Figures (49(A,B)).

To confirm that the presence of highly polymerized ribosomes were not due to random association of the monosomes, samples of polysomes were loaded after mixing with an equal volume of  $10 \text{ mg} \cdot \text{ml}^{-1}$  RNAase. Normal loading and fractionation were carried out as described above. Figure (48(A & C)) showed that the RNAase had the ability to digest and abolish the polymers of ribosomes which formed the polyribosome peaks and with a corresponding increase in the absorption observed in the monoribosome peaks.

This is consistent with the destruction of mRNA (Lin et al., 1966; Warner et al., 1966; Spirin & Gavrilova, 1969) which released ribosomes from polysomes. Presence of greater absorbance at the front of the gel which can be seen in Figure (48(A,C)) may represent ribosomal protein.

### 3.1 Ribosomes characterization in the sporangiophore

Concerning the distribution of cellular components along the sporangiophore of stage I, ribosomes were also

extracted from tip, middle and basal segments of stage I. They were equal in length (5 mm each) and in fresh weight (1 gm). The u.v. absorbance spectra were shown to be similar as those in Figure (47). The maxima and minima absorbance were at 260 and 238 nm respectively. The ratio of  $\frac{E_{260}}{E_{238}}$  and  $\frac{E_{260}}{E_{280}}$  are shown in Table (14).

A greater ratio of  $\frac{260}{280}$  of ribosomes extracted from tip

segments could be attributed to a higher purity of the ribosomes. A possible contamination with protein or with cell wall materials indicated in the  $\frac{E_{260}}{E_{238}}$  values of

ribosomes of the basal segments may also explain the variation in ratios. Another possibility is that the ribosomes of the basal segments are senescent while more active ribosomes are in the tip segments. The yields of ribosomes from tip, middle and basal segments are shown in Table (15). A greater yield of ribosomes from the tip segments of sporangiophores might indicate the specificity of accumulation of ribosomes at parts of high metabolic activity.

### 3.2 Ribosome fractionation

2.6% gels were loaded with 15 µg of ribosomes from the tip, middle and basal segments. Gels were electrophoresed and scanned as described above. Five to eight peaks of

Figure (50)

PAGE of polyribosomes extracted from tip, middle and basal segments of stage I sporangiophores and fractionated by 2.2% gels

A. Tip

1. Monomer
2. Dimer
3. Trimer
4. Tetramer
5. Pentamer
6. Hexamer
7. Septamer
8. Octamer

B. Middle

C. Basal

A 265 nm

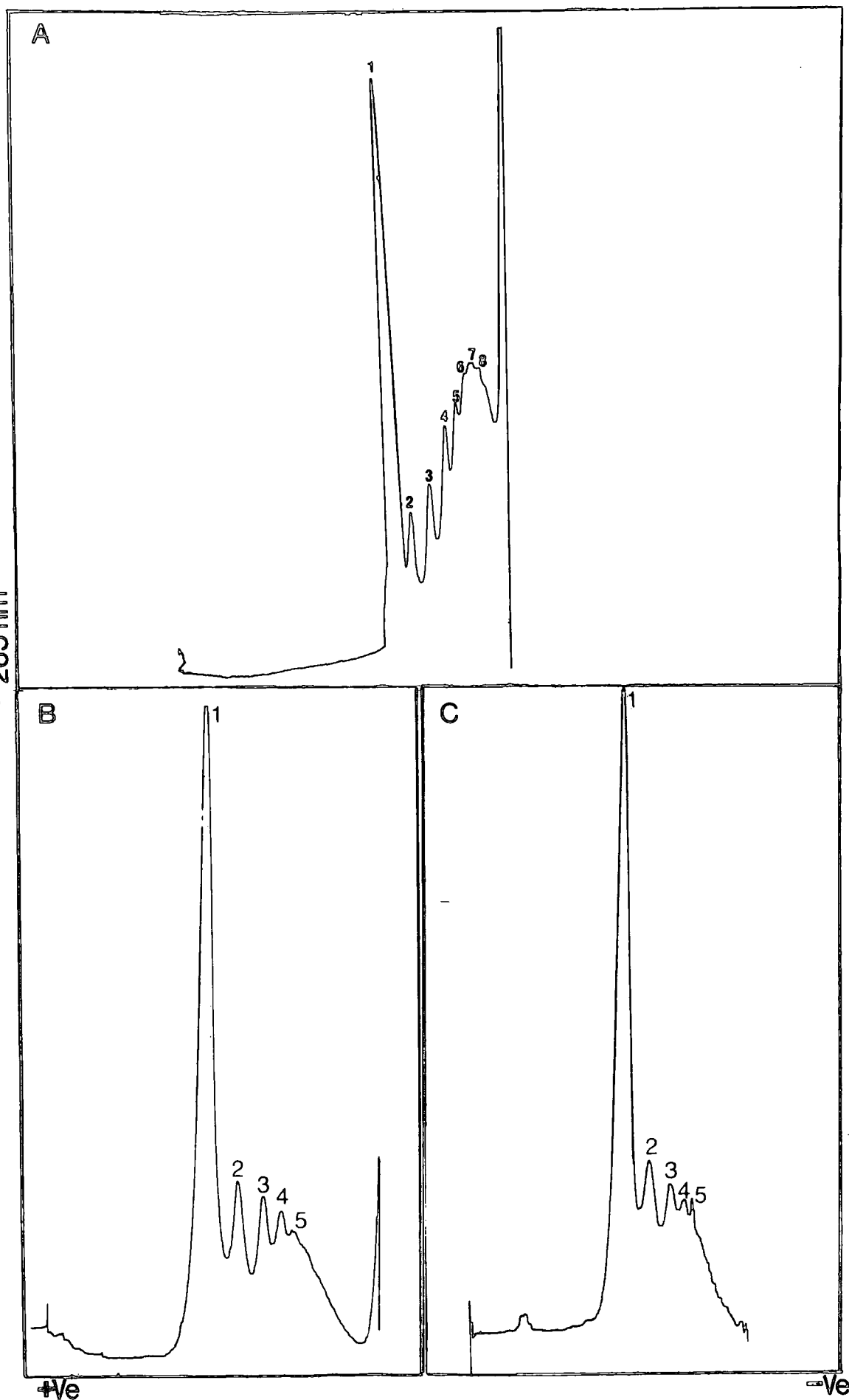


Table (14)

The ratios of  $\frac{E260}{E238}$  and  $\frac{E260}{E280}$  of ribosomes extracted from

tip, middle and basal segments

| <u>Segments</u> | $\frac{E260}{E238}$ | $\frac{E260}{E280}$ |
|-----------------|---------------------|---------------------|
| Tip             | 1.3                 | 1.8                 |
| Middle          | 1.2                 | 1.85                |
| Base            | 1.28                | 1.66                |

Table (15)

The yields of ribosomes from tip, middle and basal segments

| <u>Segments</u> | $\frac{\text{Yield of ribosomes } \mu\text{g. gm}^{-1}}{\text{fresh weight sporangiophores}}$ |
|-----------------|---|
| Tip             | 734   |
| Middle          | 343   |
| Base            | 298   |



ribosomal aggregates were regularly seen, Figure (54(A,B,C)). These were taken to represent increasing numbers of ribosomes attached to strands of m-RNA (Pearson, 1969).

Generally the peak exhibiting the greatest u.v. absorption has been designated as the monoribosome fraction with remaining peaks being successive polyribosome fractions. Hexamers and septamers and sometimes octamers were the most abundant aggregates in the profiles of ribosomes of the tip segments, Figure (51 C). Increased aggregation of the ribosomes in the tip segments indicated the possible presence of the more m-RNA which might be attributed to the higher metabolic activity of the tip region of sporangiophore in synthesizing protein for enzyme and wall production. Additionally the presence of a greater fraction of rRNA specific for the tip segments (see above) could be attributed here to the greater concentration of polyribosomes.

#### 4. Ribosomes and regeneration

During the regeneration of sporangiophores, RNA and protein were seen to be accumulated at the site of production of new wall. Such accumulation was shown to be associated with the recovery of RNA components (see qualitative study of RNA). Alternatively ribosomes were also extracted and fractionated during regeneration of

Figure (51)

PAGE of polyribosomes extracted from sporangiophores cut into tip, middle and basal segments and from intact sporangiophores during regeneration. 2.2% gels were used.

Tip

A. 2 hours

B. 8 hours

C. 12 hours

1. Monomer
2. Dimer
3. Trimer
4. Tetramer
5. Pentamer
6. Hexamer
7. Septamer

Middle

D. 2 hours

E. 12 hours

Base

F. 2 hours

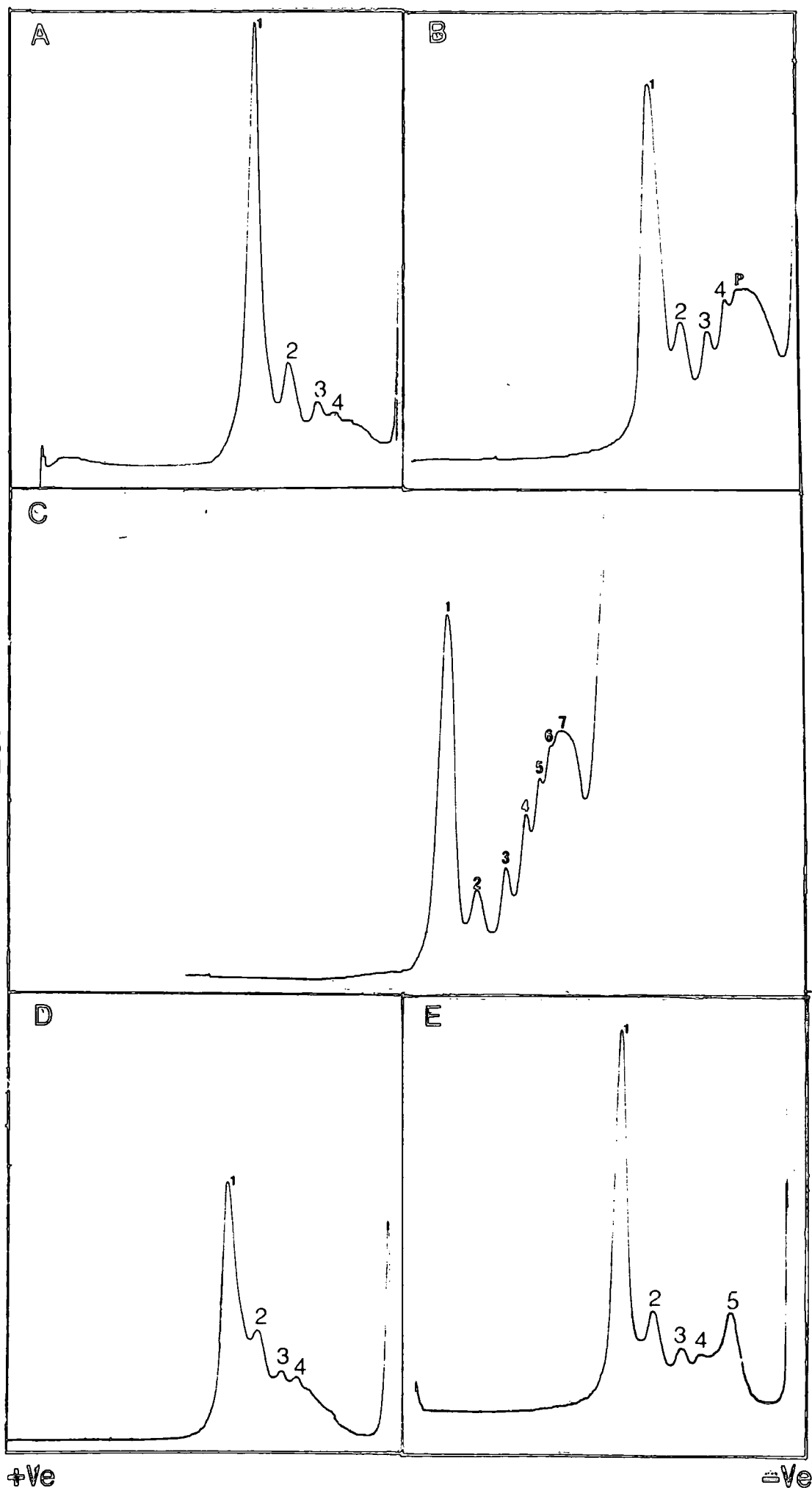
G. 12 hours

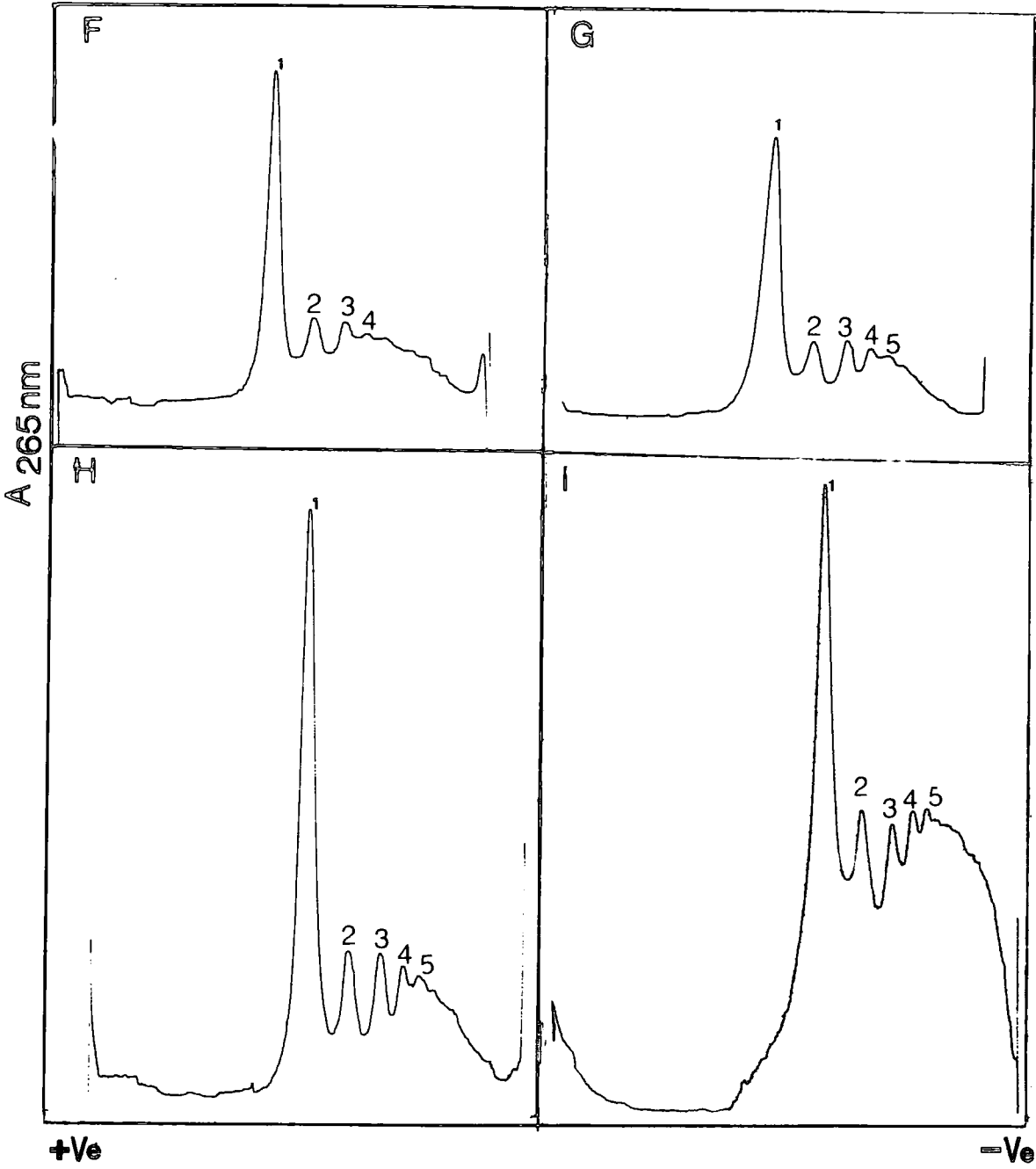
Full length sporangiophore

H. 2 hours

I. 12 hours

A 265 nm





segments, using the methods described above.

### 1. Characterization of ribosomes

The ribosomes were extracted from the tip, middle and basal segments at different intervals of time after culturing and regeneration. Methods for manipulating and culturing were similar to those described previously. U.v. absorption spectra of the ribosomes extracted from segments during the first hours of culturing (2 hours) showed a shift in the absorbance, compared with that measured at zero time. Such changes, which were found initially at 2 hours after culturing could be attributed to a possible contamination with some components released during the extraction of ribosomes which might be protein or cell wall materials. Such a shift which may possibly be attributed to contamination with protein can be used as evidence to support the initial increase of protein at the first hours of culturing of segments during regeneration (see Chapter I).

The ratios of  $\frac{E_{\max}}{E_{\min}}$  and  $\frac{E_{\max}}{E_{280}}$  of the u.v. absorption

spectra of ribosomes from segments at successive intervals are shown in Table (16). Absorbances recovered to normal values for the tip, middle and basal segments at 6, 12 and 14 hours respectively. Average yields of ribosomes of these segments were calculated using the Tester & Dure (1966)

Table (16)

The ratios of  $\frac{E_{\max}}{E_{\min}}$  and  $\frac{E_{\max}}{E_{\min}}$  of the U.V. absorption spectra of ribosomes from tip,

middle and basal segments during regeneration

|        | No. of<br>Exp. | $\frac{E_{\max}}{E_{\min}}$<br>at time (h) interval |      |      |      |      |      | $\frac{E_{\max}}{E_{\min}}$<br>at time (h) interval |      |      |      |      |     |
|--------|----------------|---|------|------|------|------|------|---|------|------|------|------|-----|
|        |                | 0   | 2    | 4    | 8    | 12   | 14   | 0   | 2    | 4    | 8    | 12   | 14  |
| Tip    | 1              | 1.28  | 0.85 | 1.1  | 1.2  | 1.25 | 1.26 | 1.45  | 1.0  | 1.4  | 1.6  | 1.71 | 1.8 |
|        | 2              | 1.44  | 1.01 | 1.3  | 1.5  | 1.49 | 1.48 | 1.7   | 1.5  | 1.55 | 1.61 | 1.69 | 1.8 |
|        | 3              | 1.22  | 0.98 | 1.21 | 1.46 | 1.51 | 1.52 | 1.76  | 1.45 | 1.5  | 1.71 | 1.81 | 1.9 |
| Middle | 1              | 1.13  | 0.78 | 0.96 | 1.0  | 1.1  | 1.2  | 1.62  | 0.99 | 1.34 | 1.4  | 1.58 | 1.6 |
|        | 2              | 1.50  | 0.81 | 0.9  | 1.01 | 1.21 | 1.4  | 1.6   | 1.2  | 1.38 | 1.64 | 1.46 | 1.5 |
|        | 3              | 1.11  | 0.91 | 1.1  | 1.05 | 1.1  | 1.15 | 1.52  | 1.1  | 1.26 | 1.39 | 1.68 | 1.6 |
| Base   | 1              | 1.1   | 0.9  | 0.69 | 1.0  | 1.1  | 1.2  | 1.4   | 0.82 | 1.3  | 1.32 | 1.57 | 1.6 |
|        | 2              | 1.3   | 0.75 | 0.98 | 1.01 | 1.11 | 1.25 | 1.8   | 1.02 | 1.26 | 1.39 | 1.6  | 1.7 |
|        | 3              | 1.4   | 0.99 | 0.98 | 1.0  | 1.2  | 1.35 | 1.78  | 1.3  | 1.3  | 1.4  | 1.49 | 1.7 |

equation where  $OD_{260} - OD_{290} = 11 = 1.0 \text{ mg.ml}^{-1}$  ribosomes tissue. Values of concentration are shown in Table (17).

As shown the recovery of normal concentrations of ribosomes of different segments were achieved at different times after cutting and culturing. The normal concentrations of ribosomes of different segments, after recovery were shown to be nearly at the same level of concentration of ribosomes extracted at 0h of each segment. Reaching a normal concentration of ribosomes appeared to be associated with the recovery of normal u.v. absorption spectra.

Recovery of ribosomes of tip segments showed to reach to a normal condition at less time compared with the recovery of ribosomal concentration of middle and basal segments. Such observations are also in agreement with the cytological and quantitative fraction studies of macromolecules in Chapter I and also appeared to be correlated with the recovery of RNA components of different regions (see RNA study). The initiation of regeneration and development of sporangiophore segments were seen to be achieved at the times of ribosomal and RNA recovery, where 6-8 hours were found for the tip segments and 12 and 14 hours were found for both middle and basal segments respectively.

Table (17)

Concentration of ribosomes  $\mu\text{g}.\text{gm}^{-1}$  fresh weight tissue  
during the regeneration

|        | <u>0h</u> | <u>2h</u> | <u>4h</u> | <u>8h</u> | <u>12h</u> |
|--------|-----------|-----------|-----------|-----------|------------|
| Tip    | 1100      | 738       | 843       | 1210      | 940        |
|        | 821       | 696       | 919       | 981       | 865        |
|        | 879       | 542       | 114       | 1101      | 710        |
| Middle | 345       | 300       | 487       | 410       | 400        |
|        | 463       | 380       | 561       | 390       | 310        |
|        | 540       | 468       | 640       | 351       | 210        |
| Base   | 298       | 198       | 256       | 385       | 319        |
|        | 362       | 248       | 364       | 489       | 308        |
|        | 499       | 398       | 481       | 597       | 398        |



## 2. Ribosomal fractionation during regeneration

Physical characterization of the ribosomes during the regeneration of the tip, middle and basal segments, were investigated using a 2.2% PAGE system, as described above. Figure (51) shows the sequence recovery of the polyribosomal fractions during the regeneration. At the initial 1 to 2 hour interval there was a loss of the high polymers of ribosomes for all of the segments, Figure (51(A, D & F)), but the level of polyribosome of tip segments was higher than those found for the middle and basal segments. A loss of polymers of ribosomes of the tip region segments was seen to be associated with an increase in the absorption of the monosome peak.

At 8 hours after culturing, the recovery of the levels of ribosomal polymers was found to be greater in the tip segments, Figure (51C), compared with those for middle segments, Figure (51E), and with those for basal segments, Figure (51G). Up to six polymers series were observed from the fractionation of the tip segments and recognized as dimer, trimer, tetramer, pentamer, hexamer and septamer, while four to five polymerized ribosomes were seen in the fractions of middle and basal segments Figure (51(E, G)). Thus a greater recovery of high polymerized ribosomes from the tip segments could be correlated with the quicker

regeneration and higher metabolic activity of this region, compared with that of the middle and basal segments.

Ribosomes of the intact sporangiophore were also fractionated during regeneration and showed subsequent recovery of high polymerized ribosomes at 6 hours during incubation, see Figure (51(H & I)). The recovery of the hexamer of the tip segments was followed by the formation of septamer and octamer on its shoulder near the front of the gel. This could be attributed to the formation of the required active polyribosomes. Consistent with this observation with RNA changes where the level of t-RNA and r-RNA increased. Recovery of the physical characteristics of the ribosomes might be correlated with the presence of active m-RNA as suggested by Srivastava (1968) and Phillips et al., (1969).

The association between regeneration and the maximum recovery of polyribosomes could also be used as an evidence for the recovery of protein synthesis associated with wall extension. This was also indicated by the accumulation of the cellular components at the site of wall healing and regeneration as found from cytological examination and by quantitative extraction.

5. Effect of RNA and protein synthesis inhibitors on the recovery of ribosomes during growth of isolated sporangiophores

Ribosomes were extracted from sporangiophores after 24 hours of culturing in cycloheximide ( $20 \mu\text{g}.\text{ml}^{-1}$ ) 6-methylpurine ( $25 \mu\text{g}.\text{ml}^{-1}$ ), rifampicin ( $25 \mu\text{g}.\text{ml}^{-1}$ ) and actinomycin D ( $25 \mu\text{g}.\text{ml}^{-1}$ ). As shown in Figure (52) there was a loss in the polymers of ribosomes of sporangiophores treated with cycloheximide, Figure (52A) and with 6-methylpurine, Figure (52B). Corresponding increases in the absorbance of monosome peaks also can be seen. Broadening of the monosome peaks may indicate an abnormal condition of ribosomes from sporangiophores treated with cycloheximide and 6-methylpurine. Thus the inhibition of growth of sporangiophores could be attributed to the inhibition of polyribosome recovery and to the abnormal condition of ribosomes, where continuous synthesis of protein at the translational level would be impaired.

Ribosomal fractions of sporangiophores treated with rifampicin, Figure (52C) and with actinomycin D, Figure (52D), showed also a loss of the polymers at 24 hours but less than that described above for cycloheximide and 6-methylpurine.

There was no inhibition of spore formation by actinomycin D and rifampicin while no spore formation was

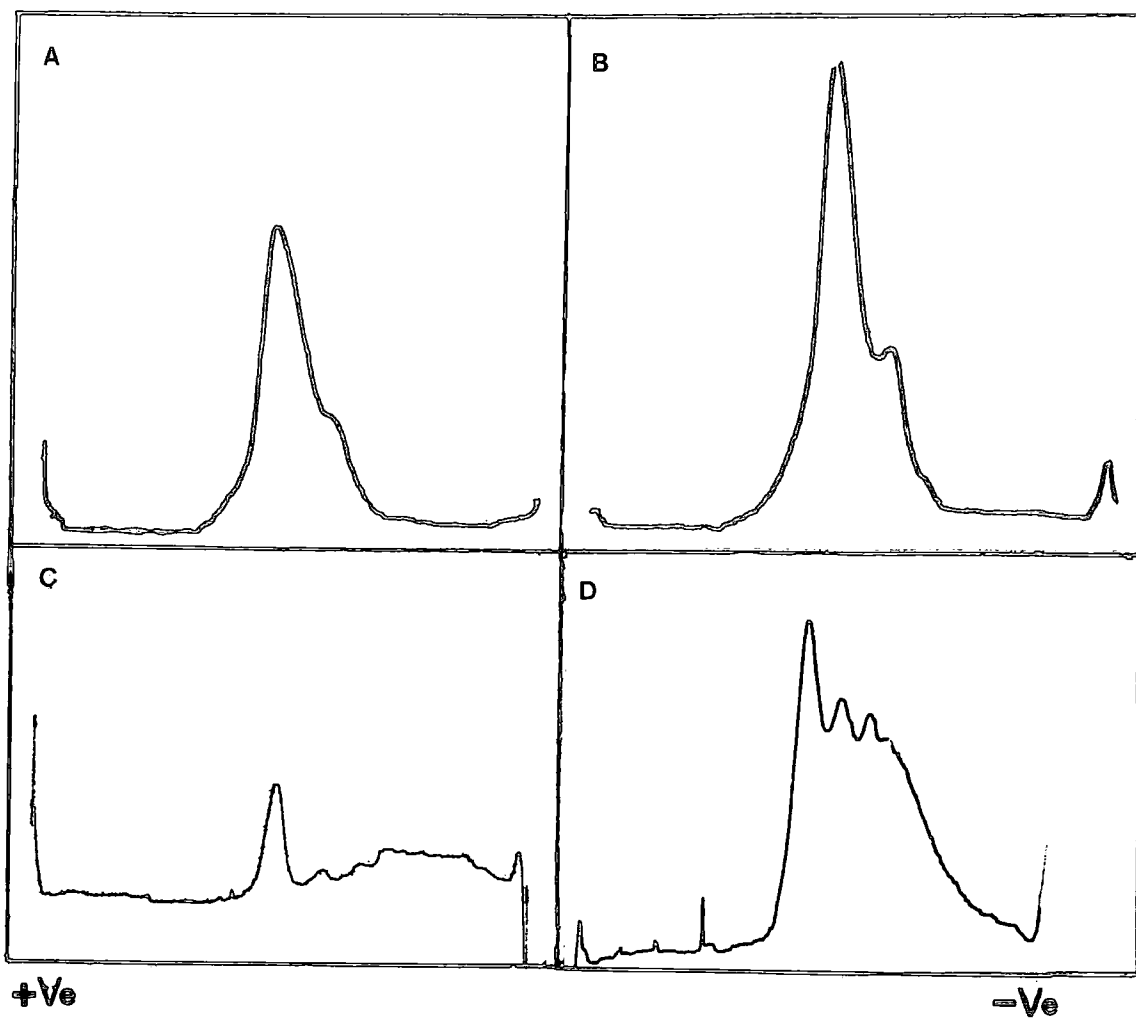
Figure (52)

PAGE of ribosomes extracted after 24 hours of growth of isolated sporangiophores in

- A. cycloheximide ( $20 \mu\text{g}.\text{ml}^{-1}$ )
- B. 6-methylpurine ( $25 \mu\text{g}.\text{ml}^{-1}$ )
- C. rifampicin ( $25 \mu\text{g}.\text{ml}^{-1}$ )
- D. actinomycin D ( $25 \mu\text{g}.\text{ml}^{-1}$ )

2.2% gels system were used

A265 nm



achieved by sporangiophores treated with cycloheximide and 6-methylpurine, since the growth was inhibited after 6 hours from the beginning of the treatment.

Therefore, it was suggested that the decrease in the final length of stage IV of sporangiophores treated with actinomycin D and rifampicin could be due to the loss of the polymers of ribosomes after spore formation. In this case the growth of the stage I sporangiophore and spore formation might be dependent on the polysomal activity with preformed mRNA. These observations also support the previous results reported in the RNA section.

#### 6. RNA extraction from ribosomes

The method described by Strangeway (1977) was employed here. It was chosen because RNA can be separated without an alcohol precipitation step, with the result that the conservation of material should be higher, and thus this was of advantage when only a small sample was available. 30  $\mu$ g and 60  $\mu$ g ribosomes were loaded onto 2.6% and 7.5% gels respectively. Similar procedures for electrophoresis and fractionation were used as for phenol-detergent prepared RNA were also employed. Figure (53) showed the fractionated species of RNA from ribosomes of the tip, middle and base segments. The results obtained were inconsistent and breakdown of the major

18S rRNA fractions frequently occurred. This may be attributed to the extraction method. Ribosome associated ribonuclease activity (Strangeway, 1977) may be partly responsible for this breakdown, which was not evident when phenol-detergent media were used. SDS however has been reported as being a mild nuclease inhibitor (Poulson, 1973). Also phenol-detergent media with high salt buffer may confer some structural stability on the RNA molecules. Similar observations were also obtained from a full length sporangiophore, Figure (54A,B).

7.5% PAGE showed greater levels of 5S and 5.85S RNA of tip segments compared with the other segment. This may be attributed to high yield of ribosomes.

Figure (53)

PAGE of high and low molecular weight RNA species extracted from polyribosomes in tip, middle and basal segments.

Tip

A. 2.6% gel

1. Unknown component
2. 18S rRNA
3. 28S rRNA

B. 7.5% gel

1. End of the gel
2. Ion front
3. Nucleotides
4. 4S RNA
5. 5S RNA

Middle

C. 2.6% gel

1. Unknown species (as above)
2. 18S rRNA
3. 28S rRNA

D. 7.5% gel

1. nucleotides
2. 4S
3. 5S

Base

E. 2.6%

1. Unknown species (see above)
2. 19S rRNA
3. 28S rRNA

F. 7.5%

1. Ion front
2. nucleotides
3. 4S
4. 5S



A<sub>265 nm</sub>

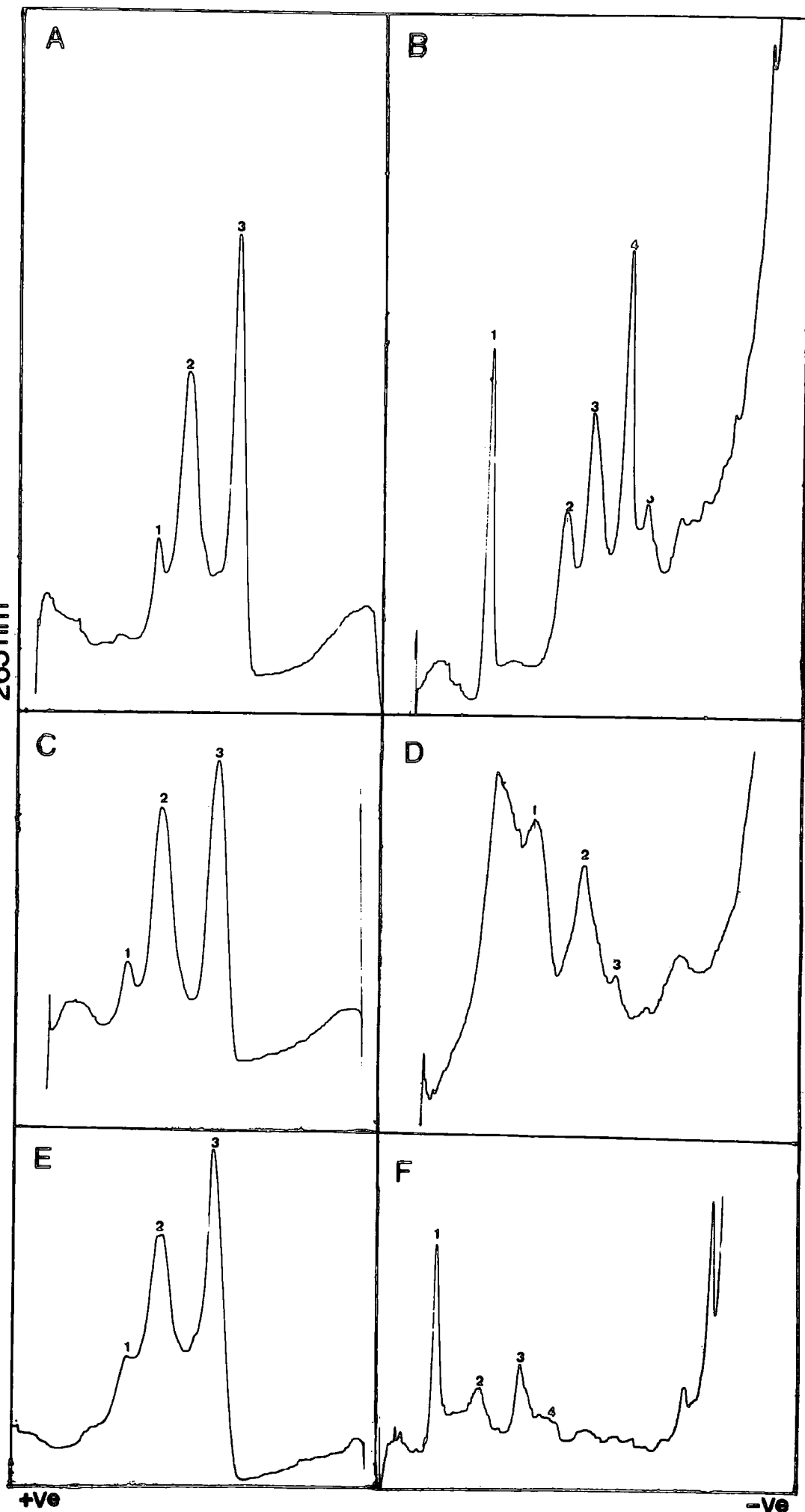


Figure (54)

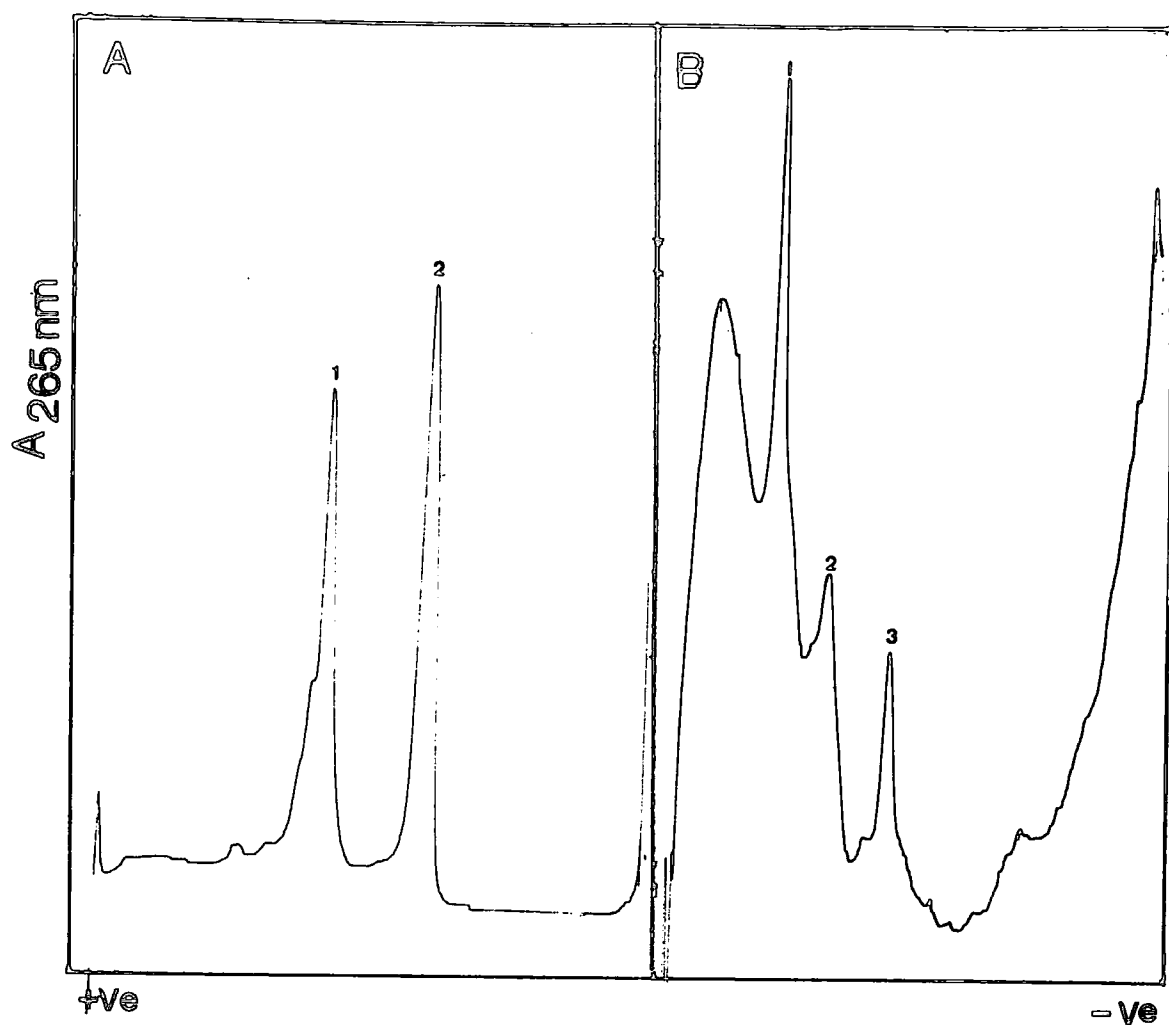
PAGE of high and low molecular weight RNA species,  
extracted from polyribosomes of full length  
sporangiophores

A - 2.6% gel

1. 18S rRNA
2. 28S rRNA

B - 7.5% gel

1. Ion front
2. nucleotides
3. 4S



## 28. PROTOPLASTS RNA

### 1. Characterization of the method

In addition to the quantitative studies of RNA from protoplast, they were also manipulated for extraction and fractionation of RNA species.

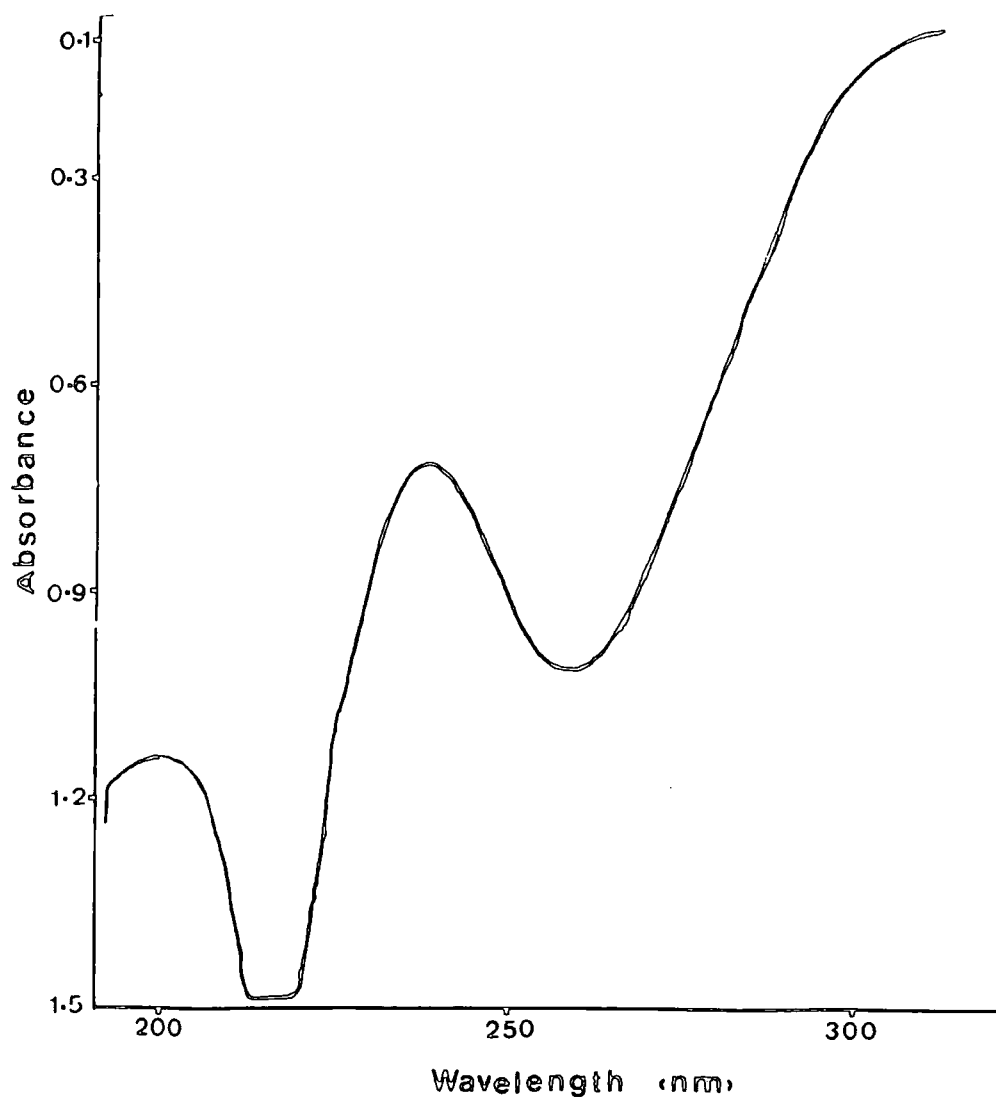
RNA was extracted by the phenol-detergent method as described previously. Isolated and stabilized protoplasts of stage I sporangiophores (1 gm fresh weight) were washed in 0.05M phosphate buffer pH 6.5 before extraction. A u.v. absorption spectrum of the RNA is shown in Figure (55). The maximum and minimum absorption were 260 and 240 nm respectively.  $\frac{E_{260}}{E_{240}}$  and  $\frac{E_{260}}{E_{280}}$  ratios were 1.28 and 1.52 respectively. The average yield of the RNA was 363 µg RNA per gm sporangiophore fresh weight.

As shown there was little difference in the ratios and u.v. absorption spectra, compared with an extract from an intact sporangiophore, see Figure (47). Also the lower concentration of the total RNA of the protoplasts could be attributed to the mechanical shock during their isolation.

Addition of the Triton X-100 to lyse the protoplasts before RNA extraction was used for a complete bursting and thus all of the RNA should be extracted. In this case most of the contents of the membrane-bound organelles were

Figure (55)

U.v. absorption spectrum of RNA extracted from protoplasts  
of full length sporangiophores



released. No differences were found between the homogenization extraction method and Triton X-100 lysed protoplast method used here. This indicated no damage by either method and was comparable with intact sporangiophore RNA, see Figure (57).

## 2. Polyacrylamide gel electrophoresis

2.6% and 7.5% polyacrylamide gel electrophoresis systems were used in fractionating the RNA species from protoplasts. Samples of 15  $\mu$ g and 30  $\mu$ g RNA were loaded onto 2.6% and 7.5% gels respectively.

Fractionations on 2.6% and 7.5% gels are shown in Figure (56). Peaks were obtained, as for intact sporangiophores with no selective breakdown or damage apparent. 5.8S RNA was also present on 7.5% gels loaded with RNA heat-treated at 60°C for 8 minutes, see Figure (56C).

### 3.1 RNA characterization during regeneration of protoplasts

RNA was extracted from protoplasts isolated from tip, middle and basal segments. The u.v. absorption spectra of the ratios of  $\frac{E_{\text{max}}}{E_{\text{min}}}$  and  $\frac{E_{\text{max}}}{E_{280}}$  absorption were obtained,

Table (18).

On incubation, the protoplast regenerated hyphae; 8, 12 and 14 hours were required for regeneration of those extracted from the tip, middle and base respectively.

Figure (56)

PAGE of high and low molecular weight RNA species,  
extracted from protoplasts released from Stage I  
sporangiophore

A. 2.6% gel

1. 18S rRNA
2. 28S rRNA
3. DNA

7.5% gels

B. Non-heat treated RNA

C. Heat-treated RNA

1. Ion front
2. nucleotides
3. 4S RNA
4. 5S rRNA
5. 5.8S rRNA



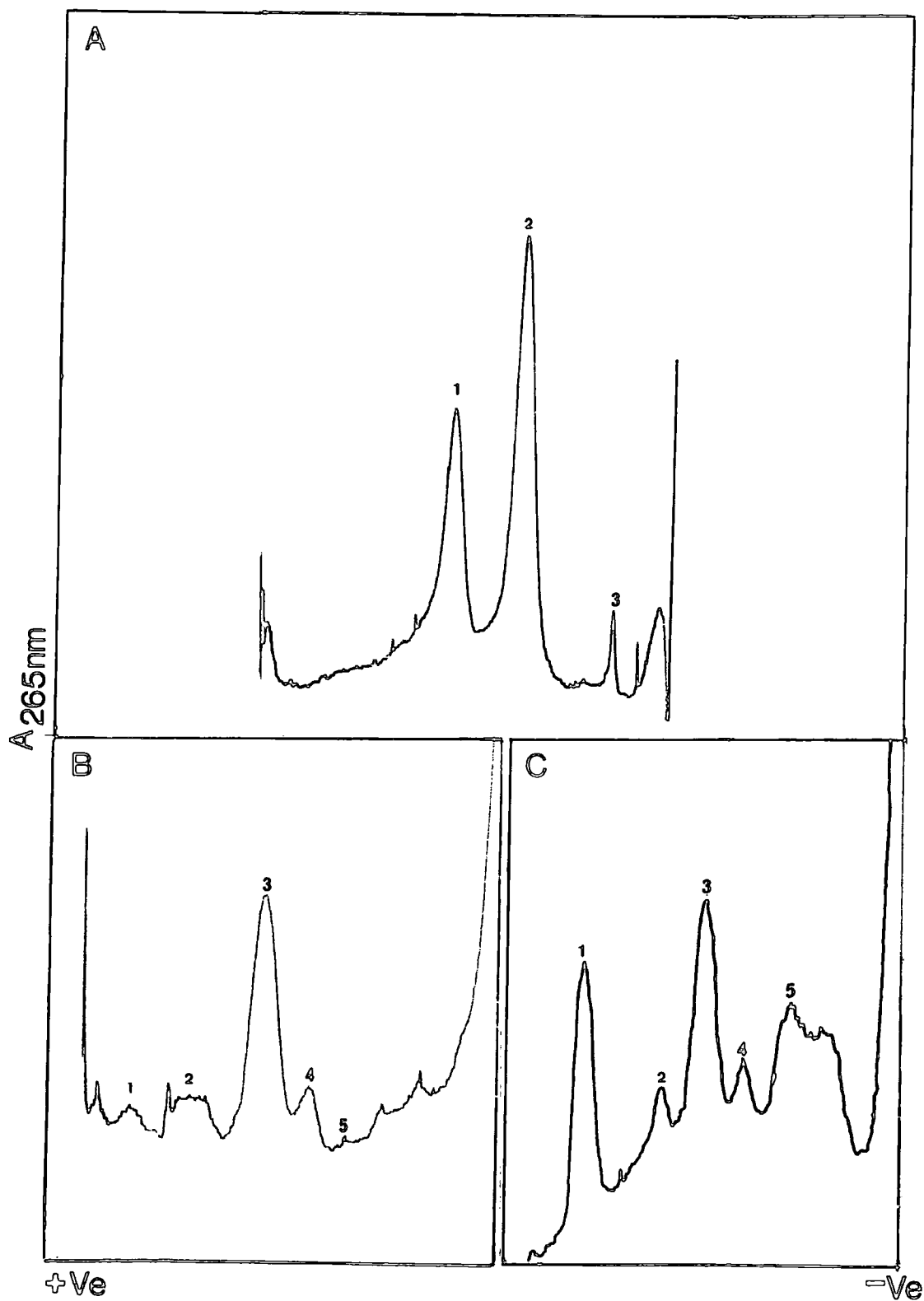


Figure (57)

PAGE of rRNA extracted from protoplasts

A. 2.6% gels

1. 18S
2. 28S
3. DNA

C. 7.5% gels

1. Ion front
2. nucleotides
3. 4S RNA
4. 5S rRNA
5. 5.8S rRNA

PAGE OF rRNA extracted from intact sporangiophores

B. 2.6% gels

1. 18S rRNA
2. 28S rRNA
3. DNA

D. 7.5% gels

1. Ion front
2. nucleotides
3. 4S RNA
4. 5S rRNA
5. 5.8S rRNA

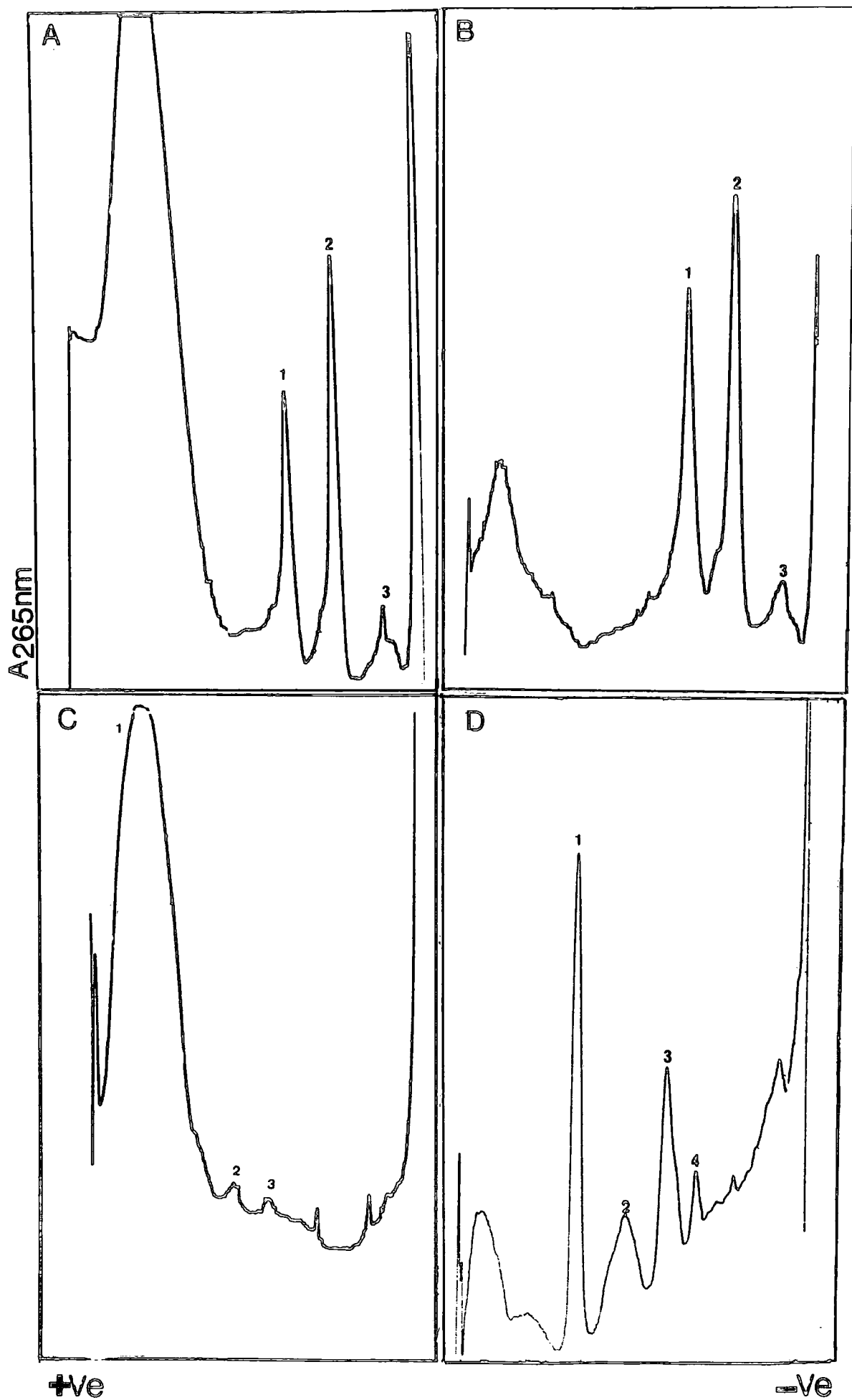


Table (18)

Characteristics of ribosomes before and after regeneration of protoplasts

Before regeneration

| <u>Protoplasts of</u> | <u>E<sub>max</sub></u><br><u>E<sub>min</sub></u> | <u>E<sub>max</sub></u><br><u>E<sub>280</sub></u> | <u>Average yield</u><br><u>μg.gm<sup>-1</sup></u> |
|-----------------------|--|--|---|
| Tip                   | 0.8  | 1.2  | 310   |
| Middle                | 1.0  | 0.95   | 120   |
| Base                  | 1.0  | 1.4  | 190   |

After regeneration

| <u>Protoplasts of</u> | <u>E<sub>max</sub></u><br><u>E<sub>min</sub></u> | <u>E<sub>max</sub></u><br><u>E<sub>280</sub></u> | <u>Average yield</u><br><u>μg.gm<sup>-1</sup></u> |
|-----------------------|--|--|---|
| Tip                   | 1.22   | 1.85   | 651   |
| Middle                | 1.3  | 1.65   | 330   |
| Base                  | 1.3  | 1.65   | 410   |

These time intervals were of the same order as for regeneration of similar segments of sporangiophores. Similar times were required to obtain a maximal accumulation of RNA (see Table 16, after regeneration) and other cellular components during initiation of regeneration (see Chapter I).

### 3.2 RNA Fractionation

RNA from the regenerated protoplasts of the tip, middle and base segments were fractionated on 2.6% and 7.5% gels. Fractionations at different intervals were obtained but Figures (58 and 59) show only the fractionations of RNA extracted from protoplasts of each segment at 2 and 10 hours.

As shown, broadening peaks of both the high and low molecular weight RNA fraction were more abundant during the initial period of protoplast incubation, see Figures (58A,C,E), 59(A,C,E)). This may be due to some breakdown of RNA species during isolation and manipulation. Breakdown products persisted longer in the regeneration of protoplasts compared with the intact segment, even so the tip protoplasts showed faster recovery. The degradation was observed to be on the total RNA rather than on a definite species. Similar observations were seen during the regeneration of the intact regions (see above).

Figure (58)

PAGE of high molecular weight RNA species extracted during regeneration of protoplasts of tip, middle and basal segments. RNA was fractionated by 2.6% gels

Tip

A. 2 hours

B. 10 hours

1. 18S rRNA

2. 28S rRNA

Middle

C. 2 hours

D. 10 hours

Basal

E. 2 hours

F. 10 hours

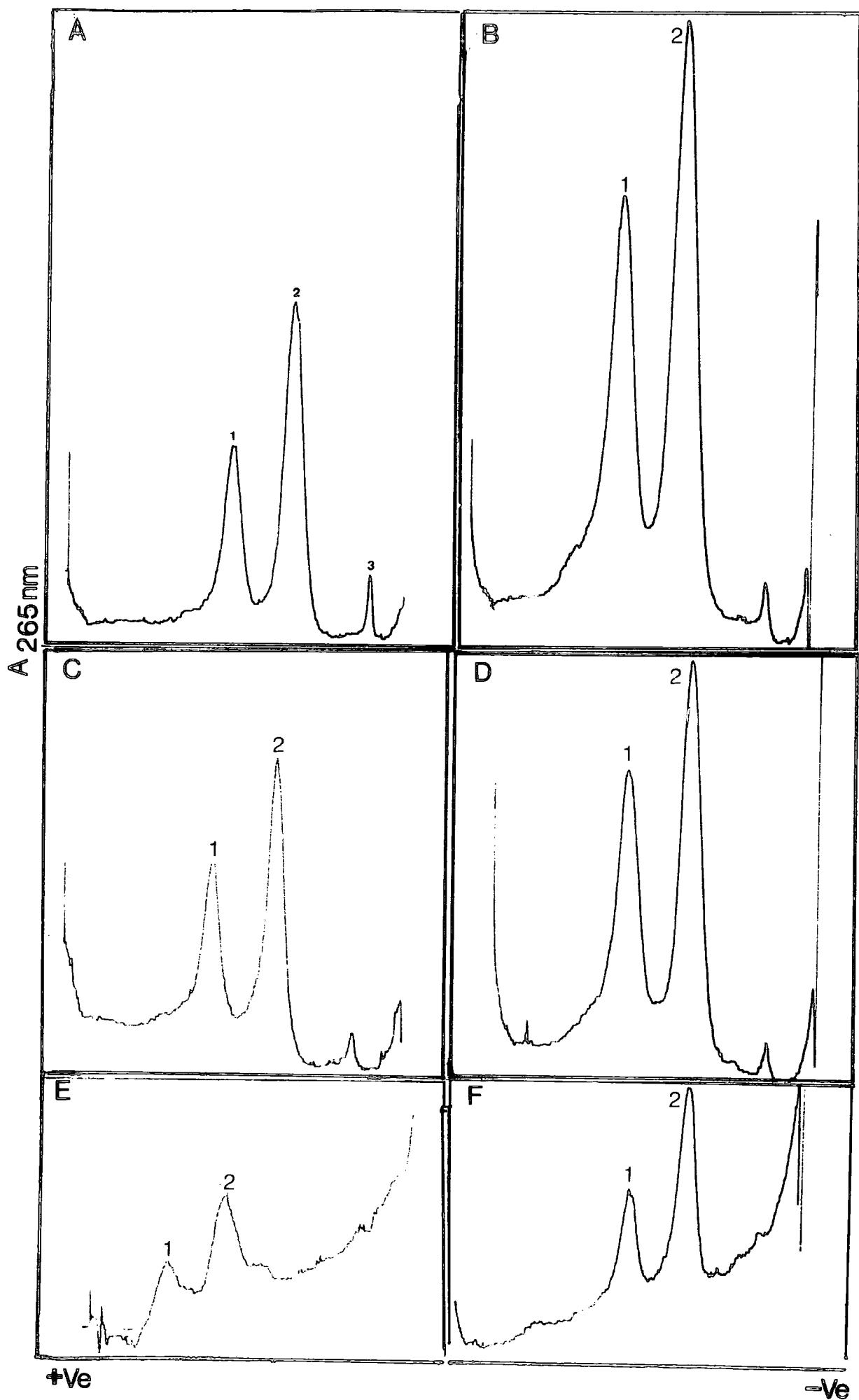


Figure (59)

PAGE of low molecular weight RNA species extracted during regeneration of protoplasts of tip, middle and basal segments. RNA were fractionated by 7.5% gels

Tip protoplasts

A. 2 hours

B. 10 hours

Middle protoplasts

C. 2 hours

D. 10 hours

Basal protoplasts

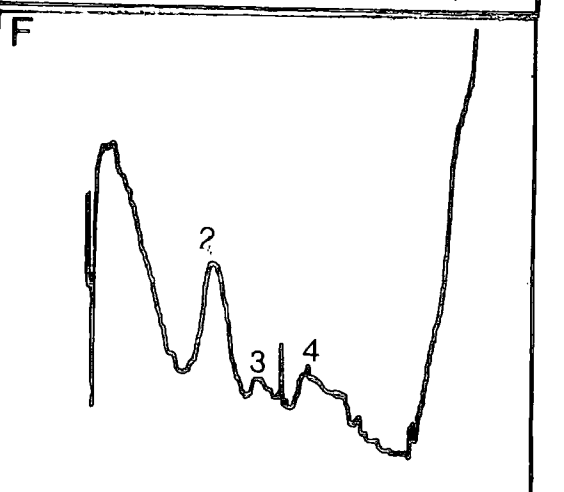
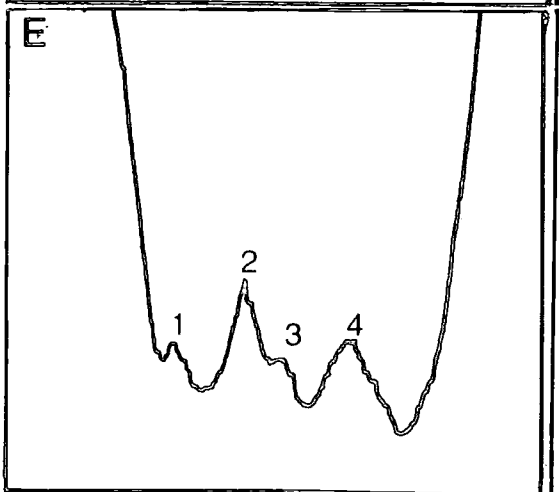
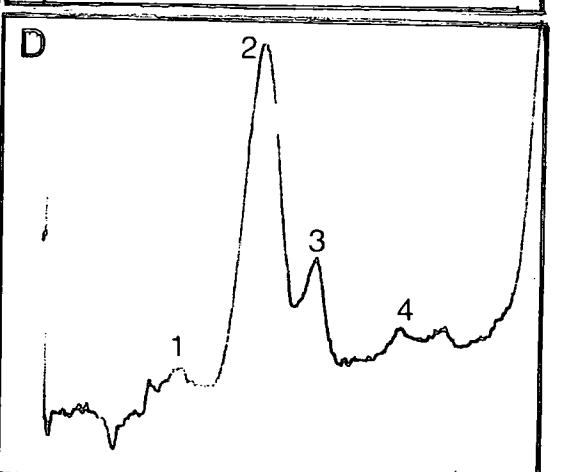
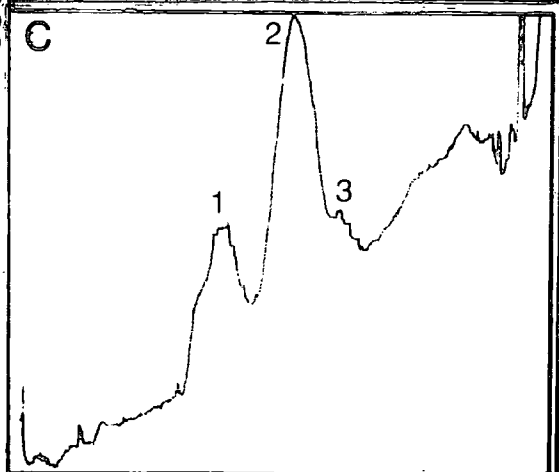
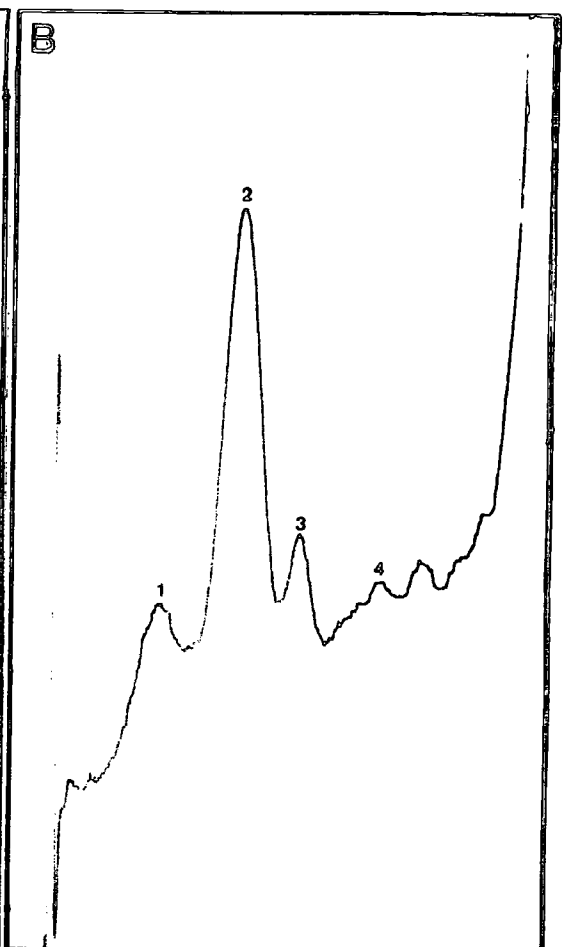
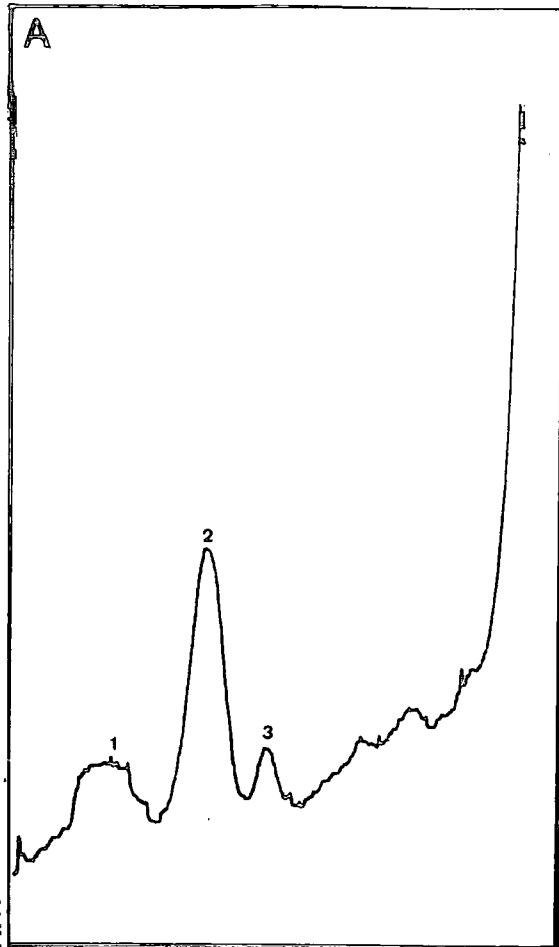
E. 2 hours

F. 10 hours

1. Nucleotides
2. 4S RNA
3. 5S RNA
4. 5.8S RNA



A<sub>265 nm</sub>



+Ve

-Ve

A good resolution of the tip protoplast RNA was achieved at 10 hours while at the same time a lower level of resolution was obtained for the middle and basal segment RNAs. The degree of recovery of RNA species appeared to be associated with pre-existing metabolic activity and was also found to be associated with the distribution of cellular components along the sporangiophore (see Chapter I).

5.8S, 5S and 4S RNA were obtained simultaneously during the recovery and regeneration, see Figure (59 (B,D,F)). This indicates that ribosomes recovery is associated with tRNA recovery. The recovery of high and low molecular weight RNA were also found during the incubation and regeneration of protoplasts isolated from intact sporangiophores, see Figure (60) of stage I. It has been observed that the initiation of the regeneration process proceeded with normal recovery of both high and low molecular weight species of RNA. These observations were also made during the regeneration of intact sporangiophore segments.

The degree of RNA recovery was also dependent on the environmental conditions. Acceleration of the recovery of RNA level was demonstrated during the incubation of the protoplasts on an agar medium (aerobic cultivation) after 1-2 hours pre-incubation in a shallow liquid medium.

Figure (60)

PAGE of RNA species extracted during regeneration of protoplasts released from full length of Stage I sporangiophores

2.6% gels

A. 2 hours

B. 10 hours

1. 18S rRNA
2. 28S rRNA

7.5% gels

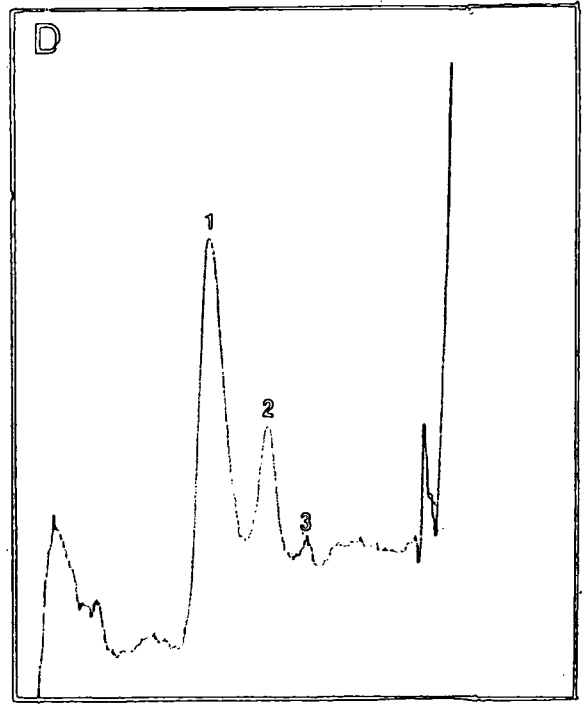
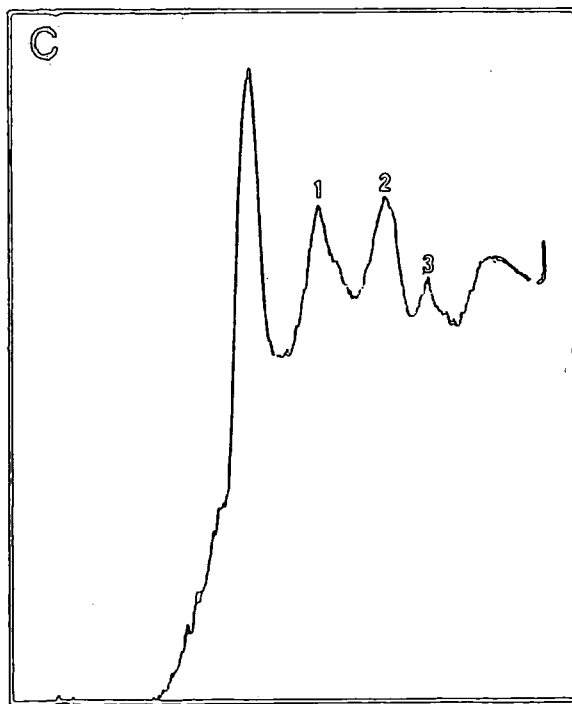
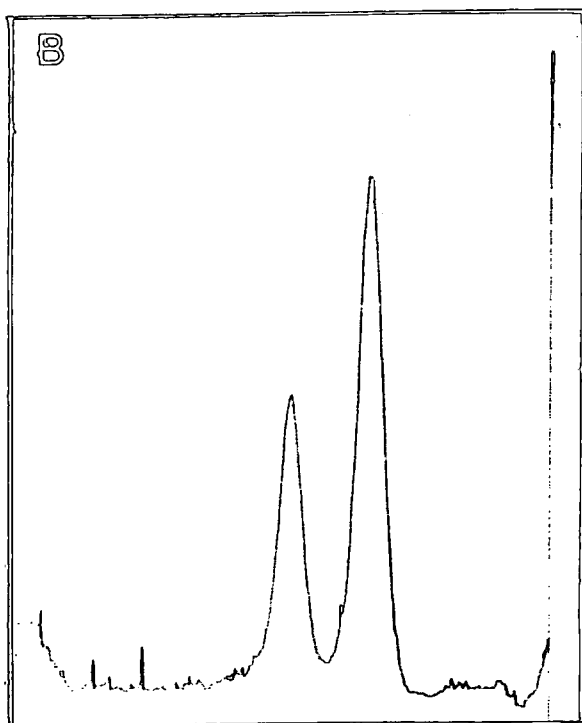
C. 2 hours

1. Nucleotides
2. 4S RNA
3. 5S rRNA

D. 10 hours

1. 4S RNA
2. 5S rRNA
3. 5.8 rRNA

A 265 nm



+Ve

-Ve

Prolonged incubation of protoplasts submerged in the liquid medium were associated with abnormal fractionation profiles and less recovery of RNA Figure (61). This could confirm the protoplasts behaviour found by cytological studies (see Chapter II).

It was suggested that wall synthesis seemed to be associated with the activity of RNA and protein synthesis. Since RNA and protein were synthesized at the same time (see review by Bramble et al., 1978) thus the protoplast regeneration and development appeared to be similar to that of spores during growth and development.

4. Effect of RNA and protein synthesis inhibitors on the RNA fractions from the regenerated protoplasts

6-methylpurine ( $25 \mu\text{g}.\text{ml}^{-1}$ ), actinomycin D ( $25 \mu\text{g}.\text{ml}^{-1}$ ) and cycloheximide ( $10 \mu\text{g}.\text{ml}^{-1}$ ) were used as potent inhibitors of RNA and protein synthesis (see page 100). The methods of manipulating and culturing were similar to those described in Chapter II. Total protoplasts isolated from full length stage I sporangiophores were used.

Figure (62) shows the effect of 6-methylpurine, Figure (62 a and b), actinomycin D (c and d) and cycloheximide (e and f) on the RNA species of protoplasts, at time of 6 and 24 hours respectively from the start

Figure (61)

PAGE of RNA species extracted after 24 hours of regeneration of protoplasts, released from a full length sporangiophore

2.6% gels

A. Non-aerated protoplasts

1. 18S rRNA
2. 28S rRNA
3. DNA

B. Aerated protoplasts

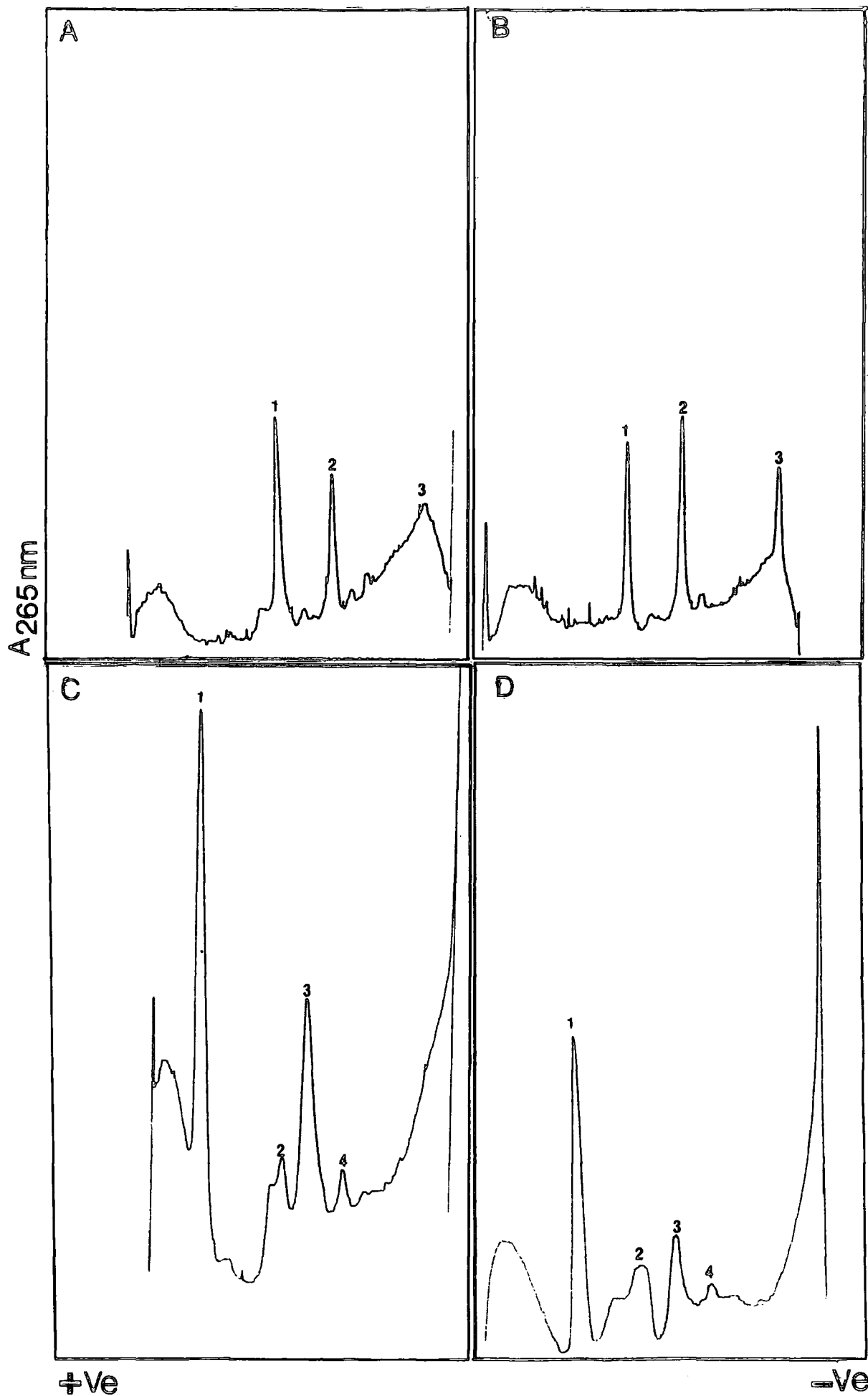
1. 18S rRNA
2. 28S rRNA
3. DNA

7.5% gels

C. Non-aerated protoplasts

D. Aerated protoplasts

1. Ion front
2. Nucleotides
3. 4S RNA
4. 5S rRNA



of regeneration. As shown there was a breakdown of the 28S and 18S rRNA species which were extracted from protoplasts treated with 6-methylpurine and cycloheximide. Greater breakdown of RNA from protoplasts treated with cycloheximide can be seen at 24 hours.

No consistent pattern of breakdown was found for the low molecular weight RNA fractions under any of the antibiotics treatment, Figure (62 (g, h, k and l)). Breakdown of the high molecular weight RNA species following treatment with 6-methylpurine and cycloheximide at 6 hours could be attributed to the inhibition of regeneration of protoplasts after that period. Likewise the reduction in the final length of the regenerated sporangiophores from the protoplasts treated with actinomycin D could be attributed to the antibiotics later effect on the RNA species.



Figure (62)

PAGE of RNA species extracted during regeneration of protoplasts which were treated with RNA and protein synthesis inhibitors

6-methylpurine ( $25 \mu\text{g}.\text{ml}^{-1}$ )

2.6% gels

a. 6 hours

b. 24 hours

7.5% gels

g. 6 hours

h. 24 hours

Actinomycin D ( $25 \mu\text{g}.\text{ml}^{-1}$ )

2.6% gels

a. 6 hours

d. 24 hours

7.5% gels

i. 6 hours

j. 24 hours

Cycloheximide ( $20 \mu\text{g}.\text{ml}^{-1}$ )

2.6% gels

e. 6 hours

f. 24 hours

7.5% gels

k. 6 hours

l. 24 hours

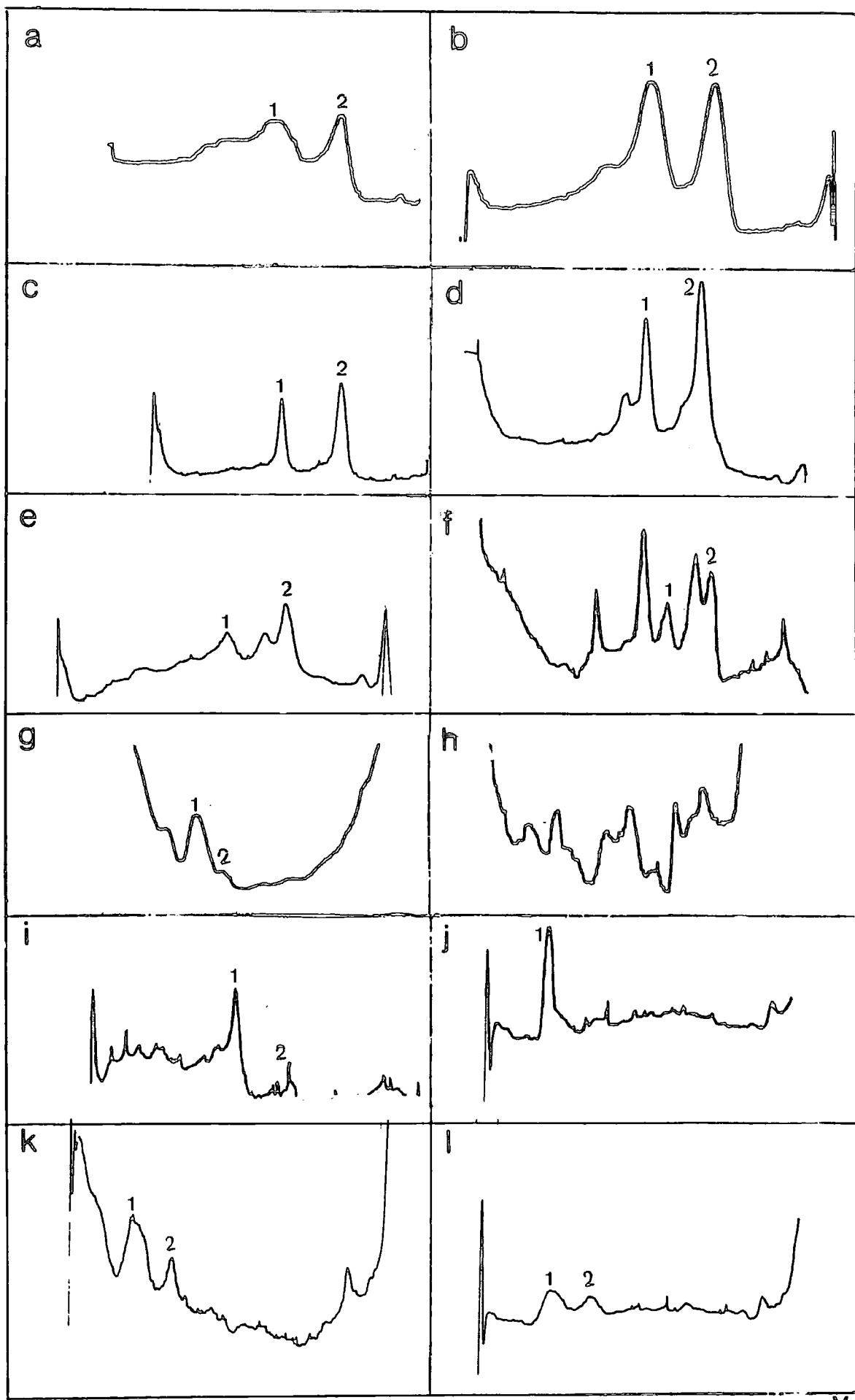
1- 18S rRNA

2- 28S rRNA

1- 4S RNA

2- 5S RNA

A 265nm



+Ve

-Ve

## 29. PROTOPLAST RIBOSOMES

### 1. U.v. characterzation

The u.v. absorption spectrum of ribosomes of proto-  
plasts isolated from stage I sporangiophore is shown in  
Figure (63). The maximum and minimum absorption wave-  
lengths were 260 nm and 238 nm respectively. The ratios  
of  $\frac{E_{\max}}{E_{\min}}$  and  $\frac{E_{\max}}{E_{280}}$  were 1.28 and 1.52 respectively. The

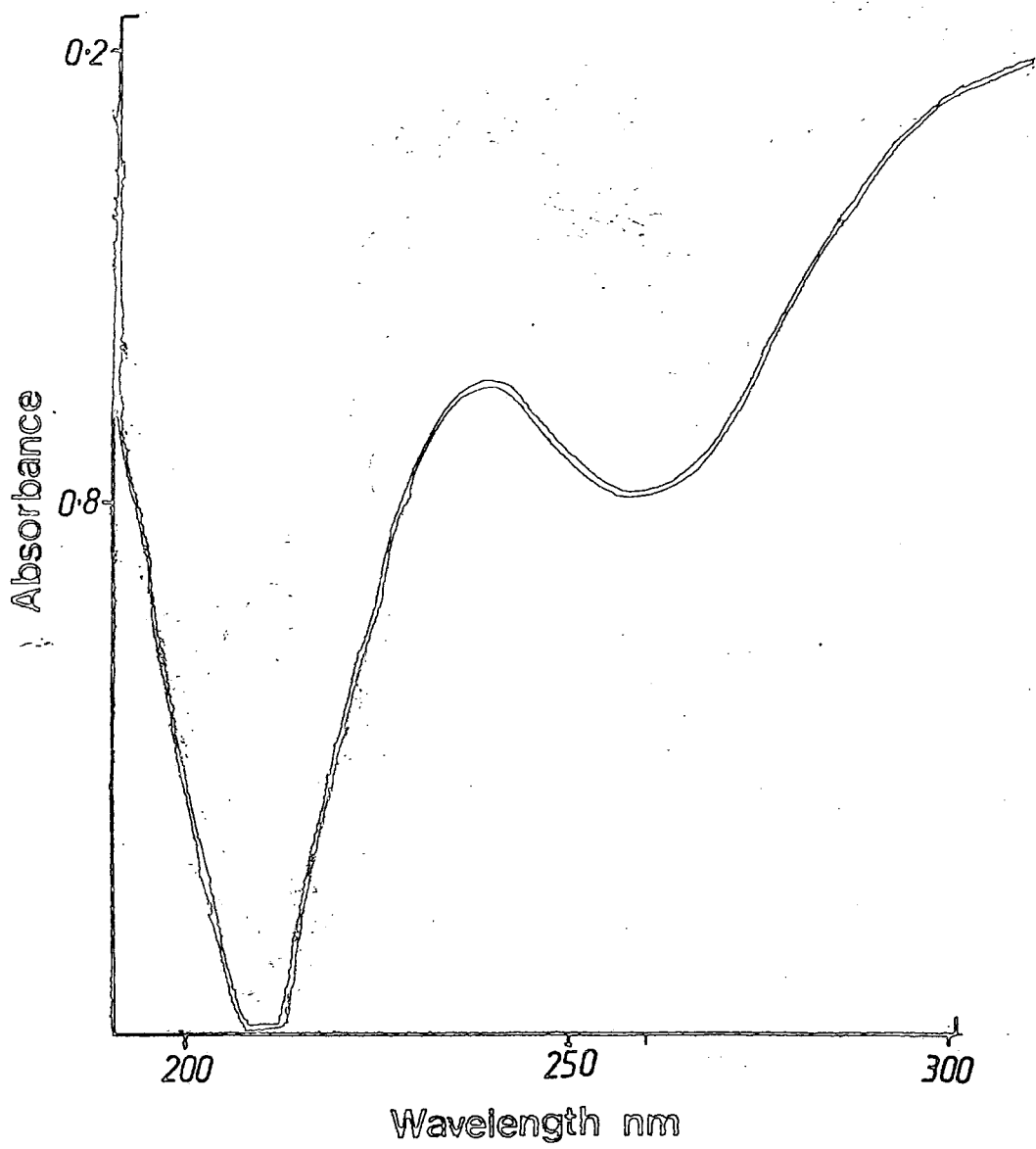
average yield of ribosomes was 620  $\mu$ g per gm fresh weight  
of the sporangiophores. As shown, there was little  
difference between the ribosomal characteristics from  
sporangiophores and protoplasts. The difference was only  
found in the total concentration of ribosomes which being  
less in the protoplast may be attributed to mechanical  
shock.

### 2. Polyacrylamide gel electrophoresis

Protoplast ribosomes were fractionated on 2.2% gels,  
the fraction profile is shown in Figure (64). As shown,  
a lower level of polymerized ribosomes can be seen for  
protoplast compared with those found in the intact sporangio-  
phore Figure (48). A series of monosome to pentamer  
polymers can be seen in the protoplasts fraction, Figure (64A).  
Mechanical shock could have contributed to such loss of the  
high polymers of ribosomes. The presence of high polymers  
was tested by adding 10 mg<sup>-1</sup> ml RNAase which digested the

Figure (63)

U.v. absorption spectrum of ribosomes extracted from  
protoplasts



highly polymerized ribosomes and correspondingly increased the absorbance of monosome peak as shown in Figure (64B).

RNA was also extracted from ribosomes of protoplasts and gave 28S, 18S for high molecular weight RNA species and 5.8S, 5S and 4S low molecular weight RNA species, Figure (65). This is similar to the situation found in an intact sporangiophore as seen above.

### 2.1 Ribosomes and regeneration of protoplasts

Protoplasts isolated from intact stage I sporangiophores were cultured using the method described for protoplasts regeneration in the RNA section (see above).

From the u.v. absorption spectrum of the ribosomes extracted at 2 hours, the ratios between  $\frac{E_{\max}}{E_{\min}}$  and  $\frac{E_{\max}}{E_{280}}$  were 1.03 and 1.02 respectively. The average yield of ribosomes was 272  $\mu\text{g}$  per gm tissue. After 8 hours of culturing the ratios of  $\frac{E_{\max}}{E_{\min}}$  and  $\frac{E_{\max}}{E_{280}}$  were 1.29 and 1.68 respectively. The average yield of ribosomes was 890  $\mu\text{g} \cdot \text{gm}^{-1}$ .

The lower values of the ratios at the first 2 hours could be attributed to a possible contamination with extraneous substances and also possibly due to an extension of the mechanical effect. The recovery of the ribosomes absorbance and concentration was seen to be associated with the initiation of regeneration. Such

Figure (64)

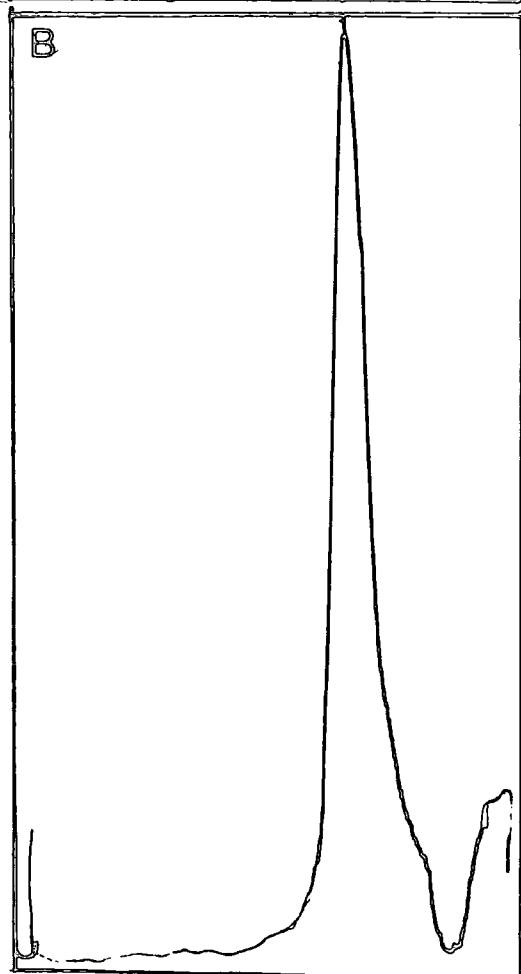
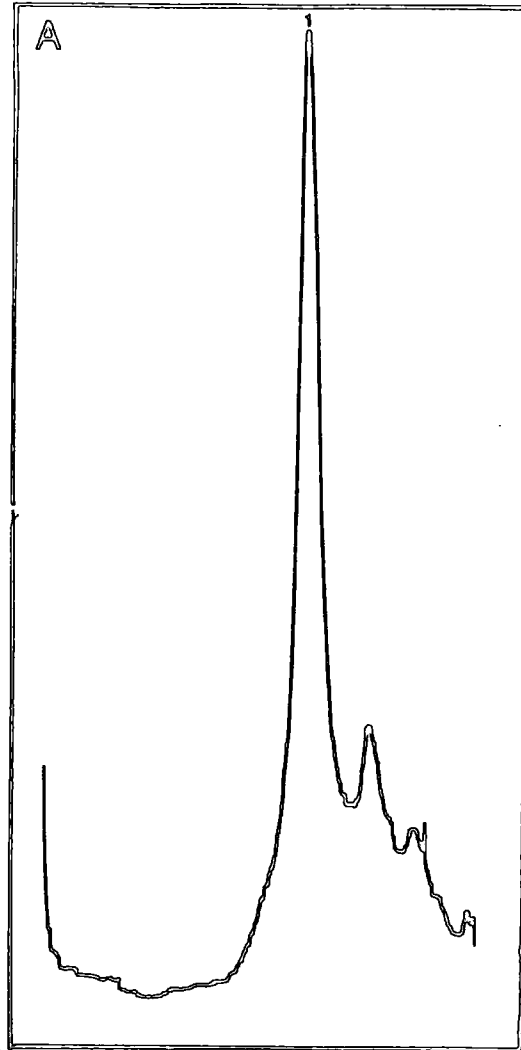
PAGE of polyribosomes extracted from protoplasts of sporangiophores and fractionated by 2.2% gels

A. Normal fractionation

B. Fractionation shows polyribosomes after addition of  $10 \text{ mg.ml}^{-1}$  RNAase

1- Monosomes

A 265nm



+Ve

-Ve



Figure (65)

PAGE of rRNA extracted from polyribosomes of protoplasts by the SDS method. RNA were fractionated by 2.6% and 7.5% gels

A. 2.6% gels

1. 18S
2. 28S

7.5% gels

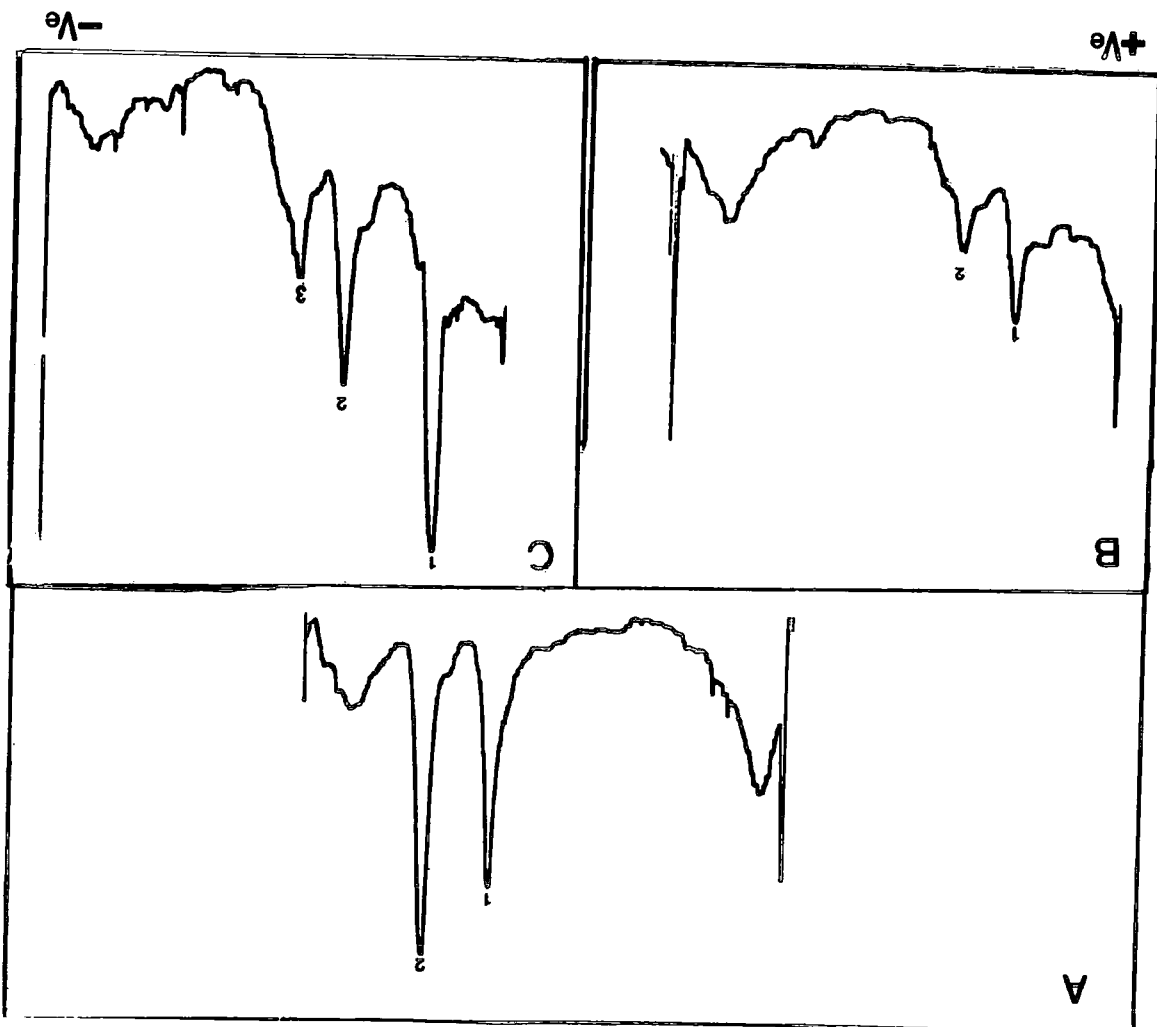
B. Non-heated treated RNA

1. 4S rRNA
2. 5S rRNA

C. Heat-treated RNA

1. 4S rRNA
2. 5S rRNA
3. 5.8S rRNA

A 265nm



recovery resembled that found for the RNA species during the regeneration of protoplasts.

The recovery of ribosomes was seen to be dependent on the media used for regeneration, since the media with limited aerobic condition gave poor regeneration. Likewise this was found to impair the recovery of ribosomal concentration, see for other living systems (Lin et al., 1967; Harvey, 1973; Mirkes, 1974; Roheim et al., 1974).

Using 2.2% gel electrophoresis system ribosomes from protoplasts which were pre-incubated in a shallow layer of liquid medium and then on solid media gave a greater level of polymers of ribosomes, Figure (66), while those cultured submerged in limited aerobic conditions gave less polymers and less regeneration, Figure (67). Thus the quicker recovery and regeneration of protoplasts which were cultured in a shallow liquid medium and then on to the surface of the agar (Chapter II) may be attributed here to greater recovery of ribosomes. So the recovery of ribosomes can be used here as evidence for determining the activity and regeneration of protoplasts.

The method which was shown to be suitable for ribosomal recovery and shown to give a good regeneration was used in the following experimental sets.

Figure (66)

PAGE of polyribosome extracted at different intervals during regeneration of protoplasts on solid medium. Protoplasts were pre-incubated in liquid medium for 1-2 hours. Polyribosomes were fractionated by 2.2% gels

- A. 1 hour
- B. 2 hours
- C. 6 hours
- D. 12 hours

p- polyribosomes

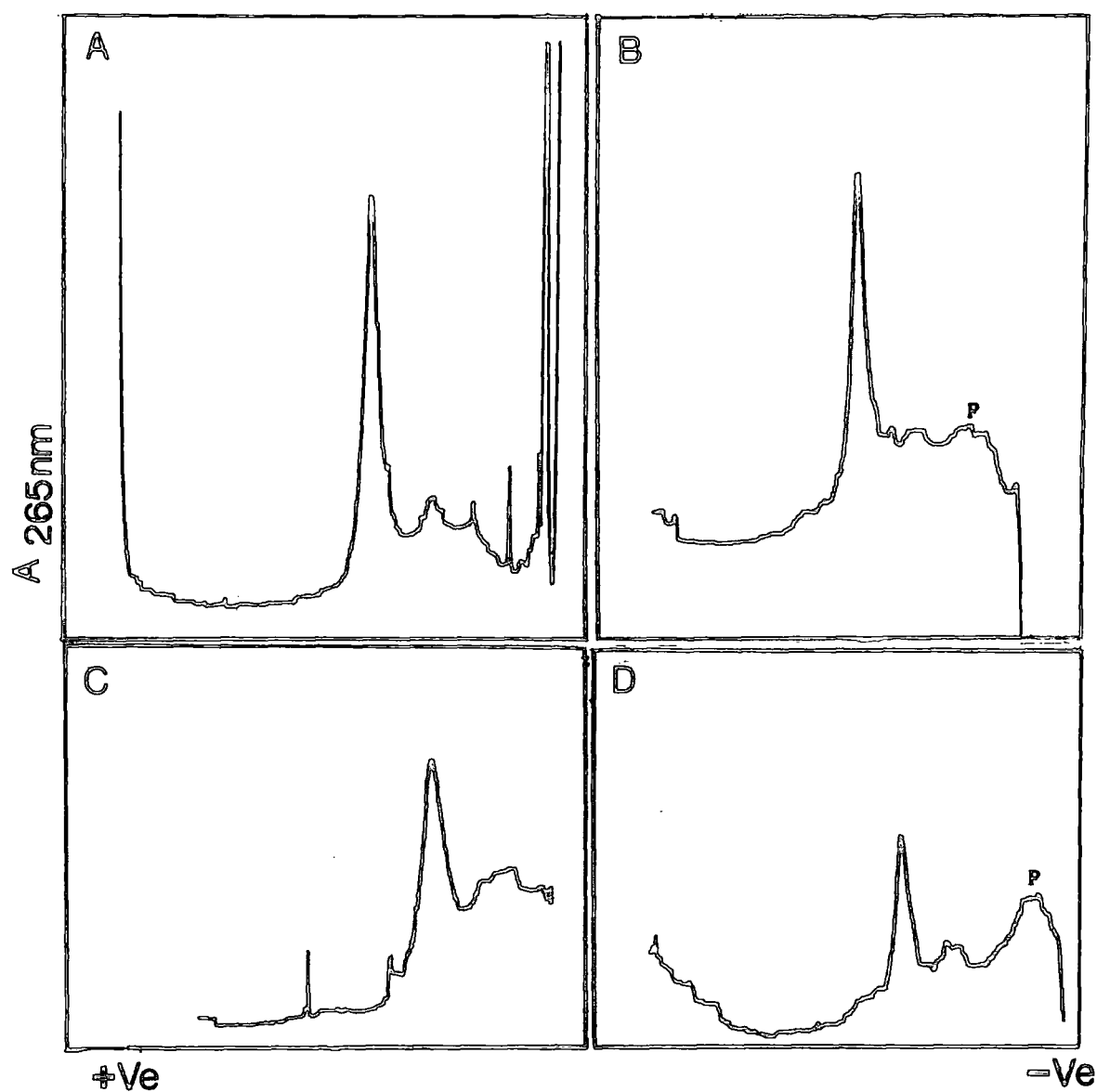


Figure (67)

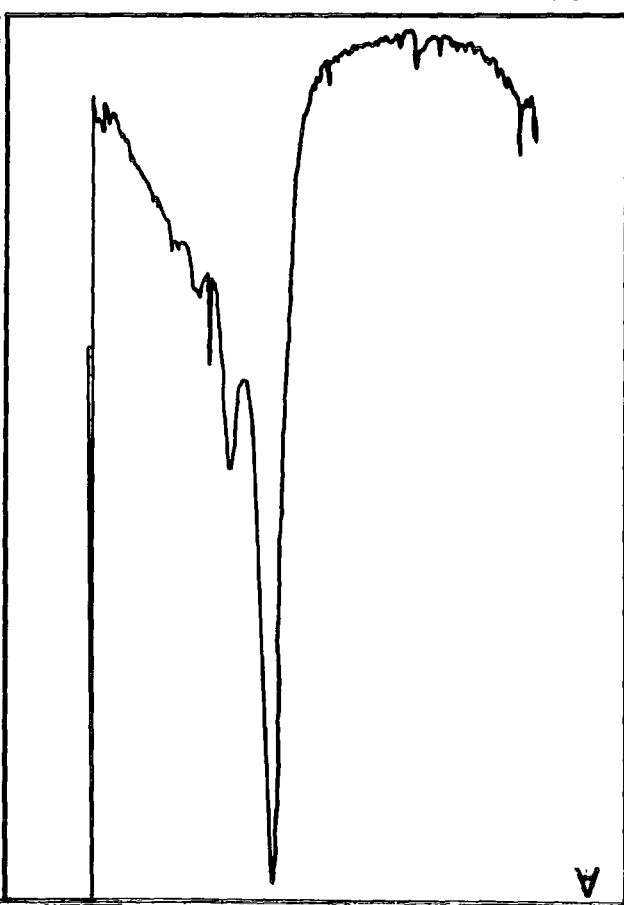
PAGE of polyribosomes extracted at different intervals during regeneration of protoplast in liquid medium. 2.2% gels were used.

A. 2 hours

B. 12 hours

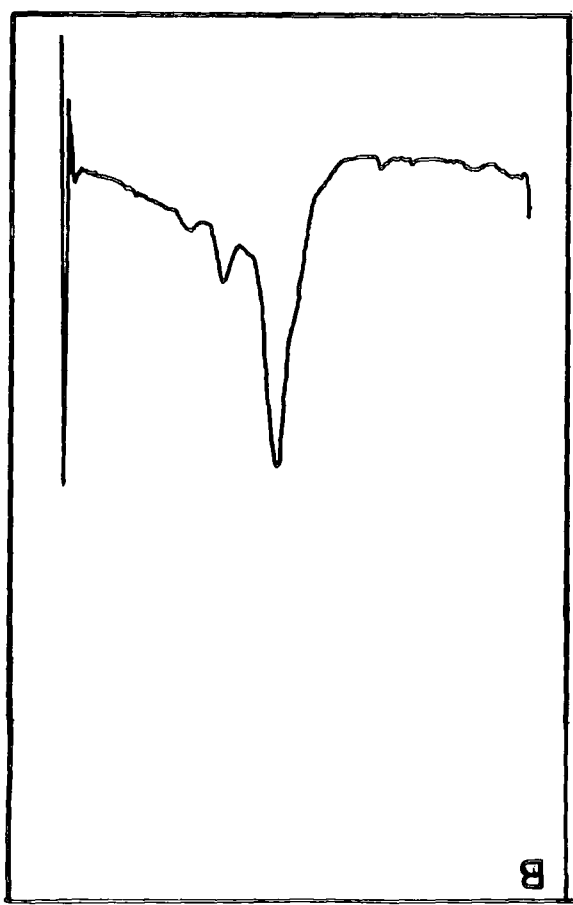
A 265 nm

+Ve



A

-Ve



B

## 2.2. Fractionation of ribosomes extracted from protoplasts of different segments during regeneration

Protoplasts isolated from segments which were of equal length (5 mm) and fresh weight (1 gm) were incubated in the above regeneration medium (see Chapter II). Ribosomes were extracted at intervals from regenerating protoplasts of different segments and 15  $\mu$ g ribosomes was usually used as a suitable loading on 2.2% gels. During the first hours of culturing, there was found to be a loss in the polymers of ribosomes from protoplasts of different segments. The recovery of polyribosomes was shown to be initiated at 6 hours in the tip protoplasts while at 12 and 14 hours after culturing, in the middle and basal protoplasts, Figures (68, 69, 70).

The recovery of polyribosomes from protoplasts of different segments appeared to be associated with decreasing absorbance of the monoribosome peaks. At the time of polyribosomal recovery the regenerations of the protoplasts were seen, initiated. The tip protoplast started to regenerate before the others. Therefore the quicker recovery of polyribosomes and regeneration both appeared to be associated. Greater activity of the tip region for regeneration and growth could be interpreted here as a greater activity of their ribosomes to form polyribosomes. Likewise the maximal recovery of polyribosomes appeared to be also in association with the



Figure (68)

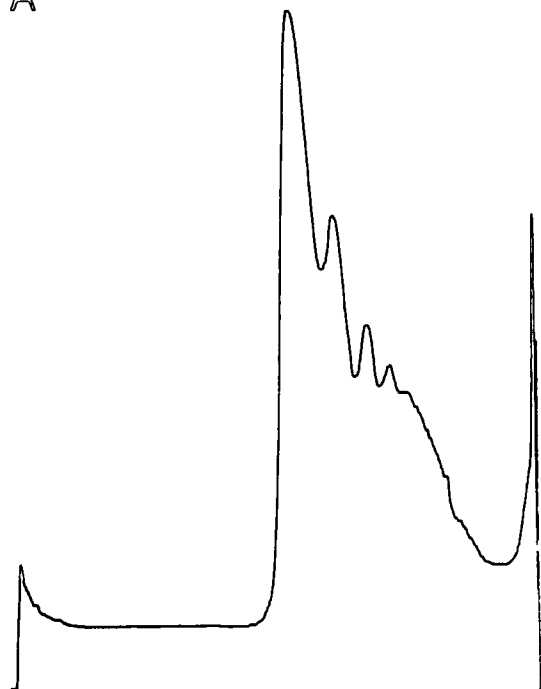
PAGE of polyribosomes extracted at different intervals during the regeneration of protoplasts, released from the tip segments of stage I sporangiophores. Polyribosomes were fractionated by 2.2% gels. Culture method was as for (67)

- A. 2 hours
- B. 4 hours
- C. 6 hours
- D. 8 hours

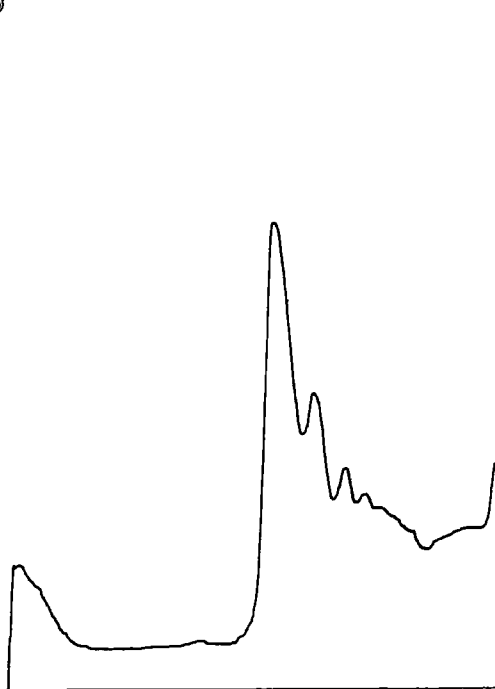
p - polyribosomes

A 265nm

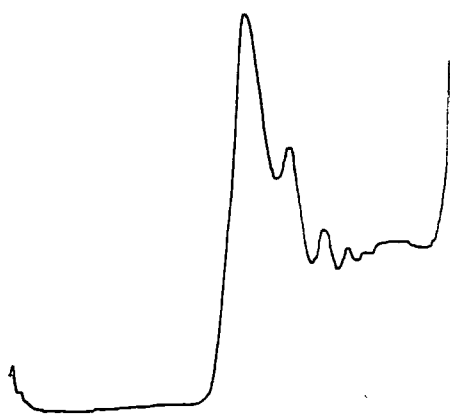
A



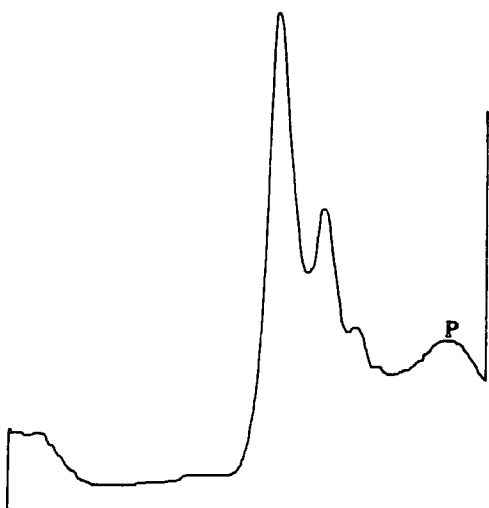
B



C



D



+Ve

-Ve

Figure (69)

PAGE of polyribosomes extracted during the regeneration of protoplasts, released from middle segments of stage I sporangiophores. Polyribosomes were fractionated by 2.2% gels. Culture method was as for (67)

- A. 2 hours
- B. 12 hours
- C. 24 hours

p - polyribosomes

A 265 nm

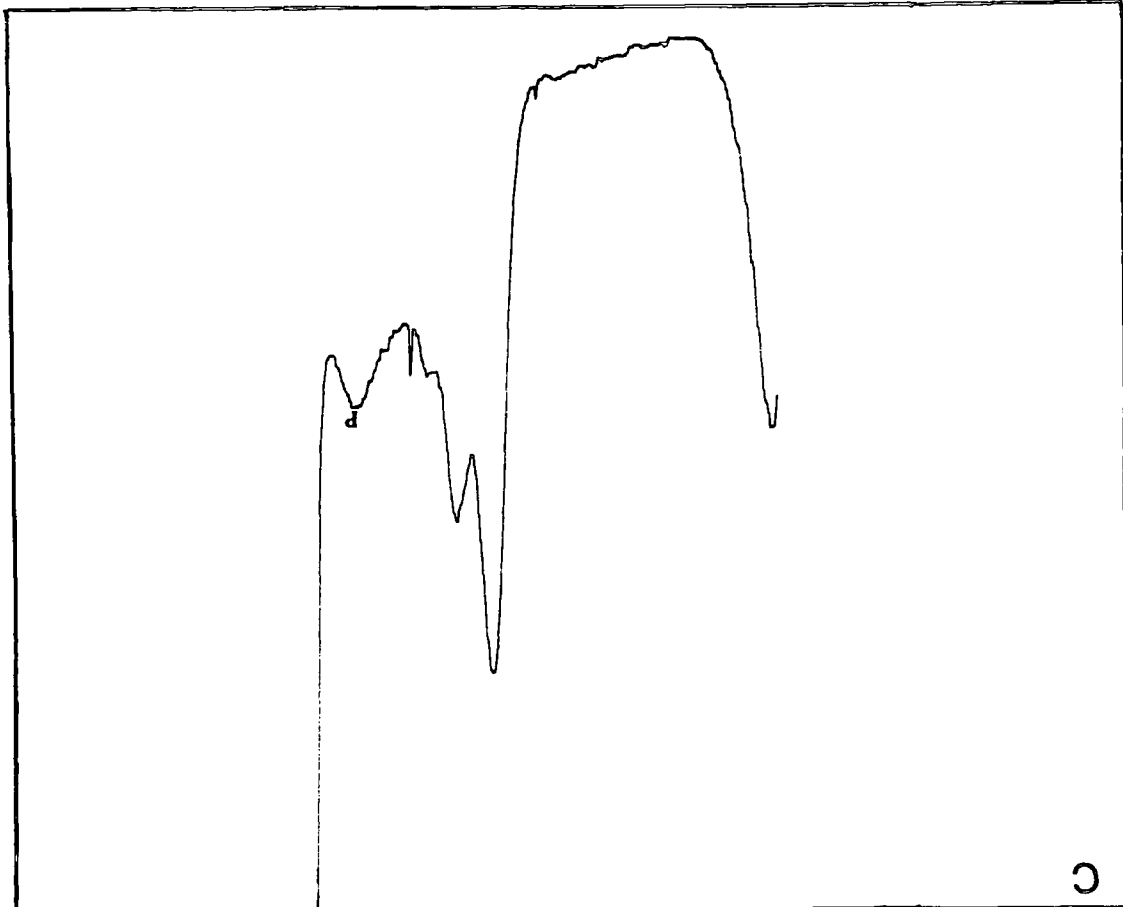
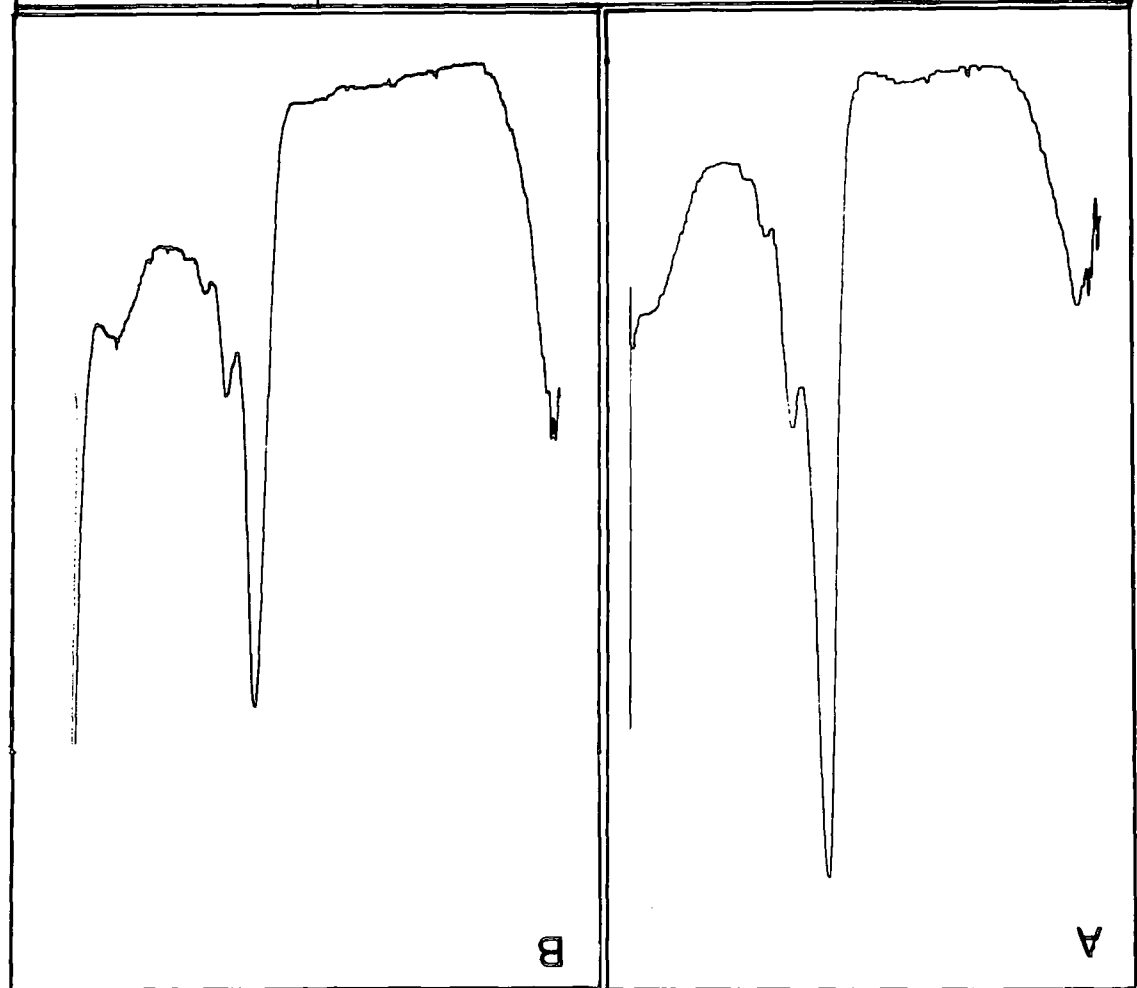


Figure (70)

PAGE of polyribosomes extracted during the regeneration of the protoplasts released from the basal segments of stage I sporangiophores. Polyribosomes were fractionated by 2.2% gels. Culture method was as for (67)

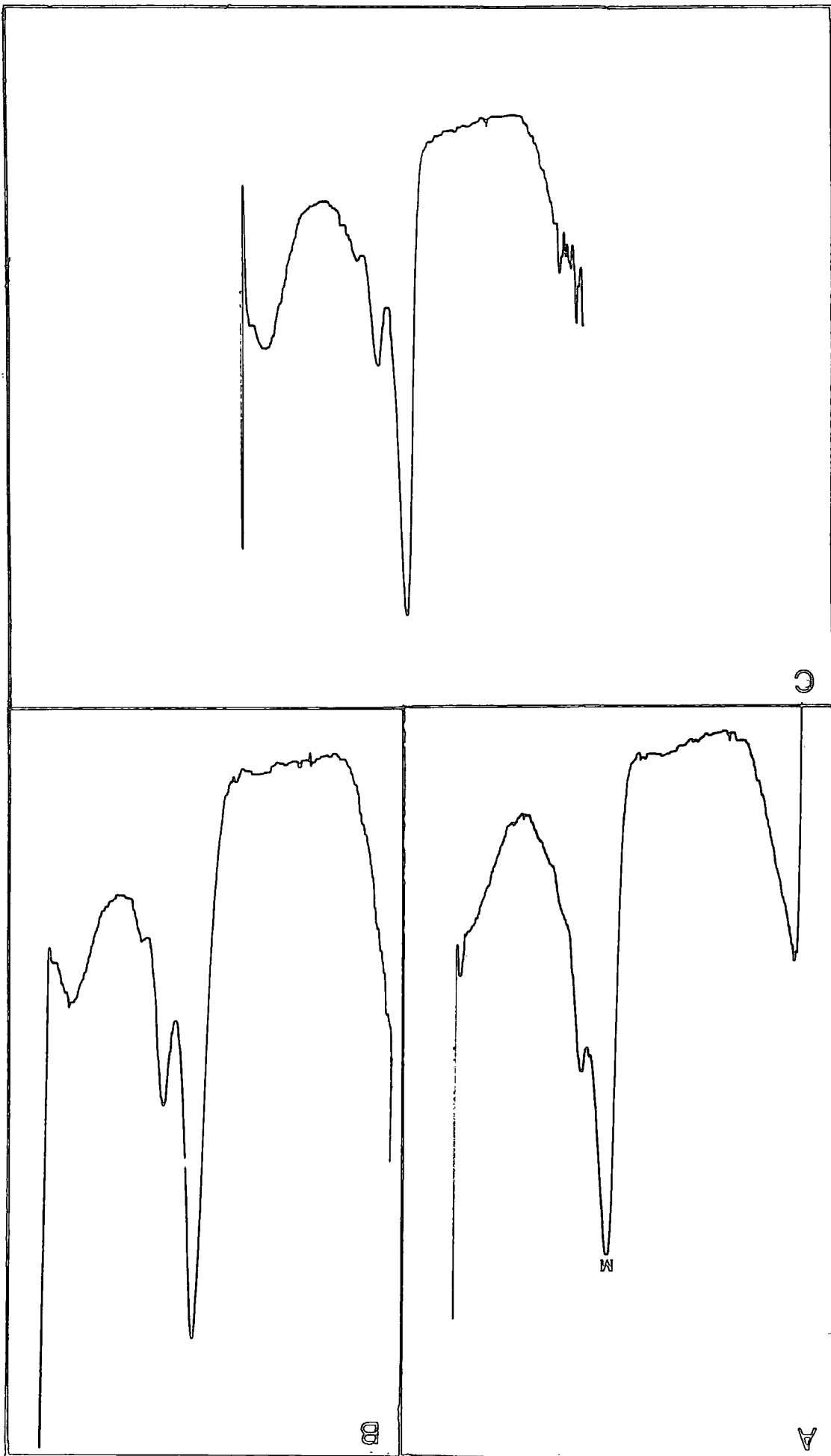
- A. 2 hours
- B. 12 hours
- C. 24 hours

M - Monosomes

A 265 nm

+Ve

-Ve



recovery of RNA species.

Similar observations of recovery and regeneration were seen for the protoplasts isolated from intact sporangiophores, Figure (71), where highly polymerized ribosomes were obtained at 8 hours.

### 3. Effect of RNA and protein synthesis inhibitors on ribosomes during regeneration of protoplasts

Protoplasts isolated from intact stage I sporangiophores were treated with 6-methylpurine ( $26 \mu\text{g}.\text{ml}^{-1}$ ), actinomycin D ( $25 \mu\text{g}.\text{ml}^{-1}$ ) and cycloheximide ( $20 \mu\text{g}.\text{ml}^{-1}$ ). Protoplasts which were grown in normal medium was used as a control.

There was no regeneration found after 6 hours by the protoplasts incubated in cycloheximide or 6-methylpurine but there was a regeneration by the protoplasts which were cultured in actinomycin D. Ribosomes were extracted and fractionated after 24 hours of culturing and the results are shown in Figure (72).

The effect of cycloheximide in inhibiting the growth of protoplasts can be attributed to the breakdown of the ribosomes into subunits, Figure (72C). Breakdown in synthesizing protein (Spirin & Gavrilova, 1969; Hadholova & Nikolaev, 1976). The breakdown of the RNA which was found above could possibly be attributed here

Figure (71)

PAGE of polyribosomes extracted at different intervals during regeneration of protoplasts released from stage I sporangiophores. Polyribosomes were fractionated by 2.2% gels. Protoplasts suspension were cultured on the solid medium

A. 2 hours

B. 8 hours

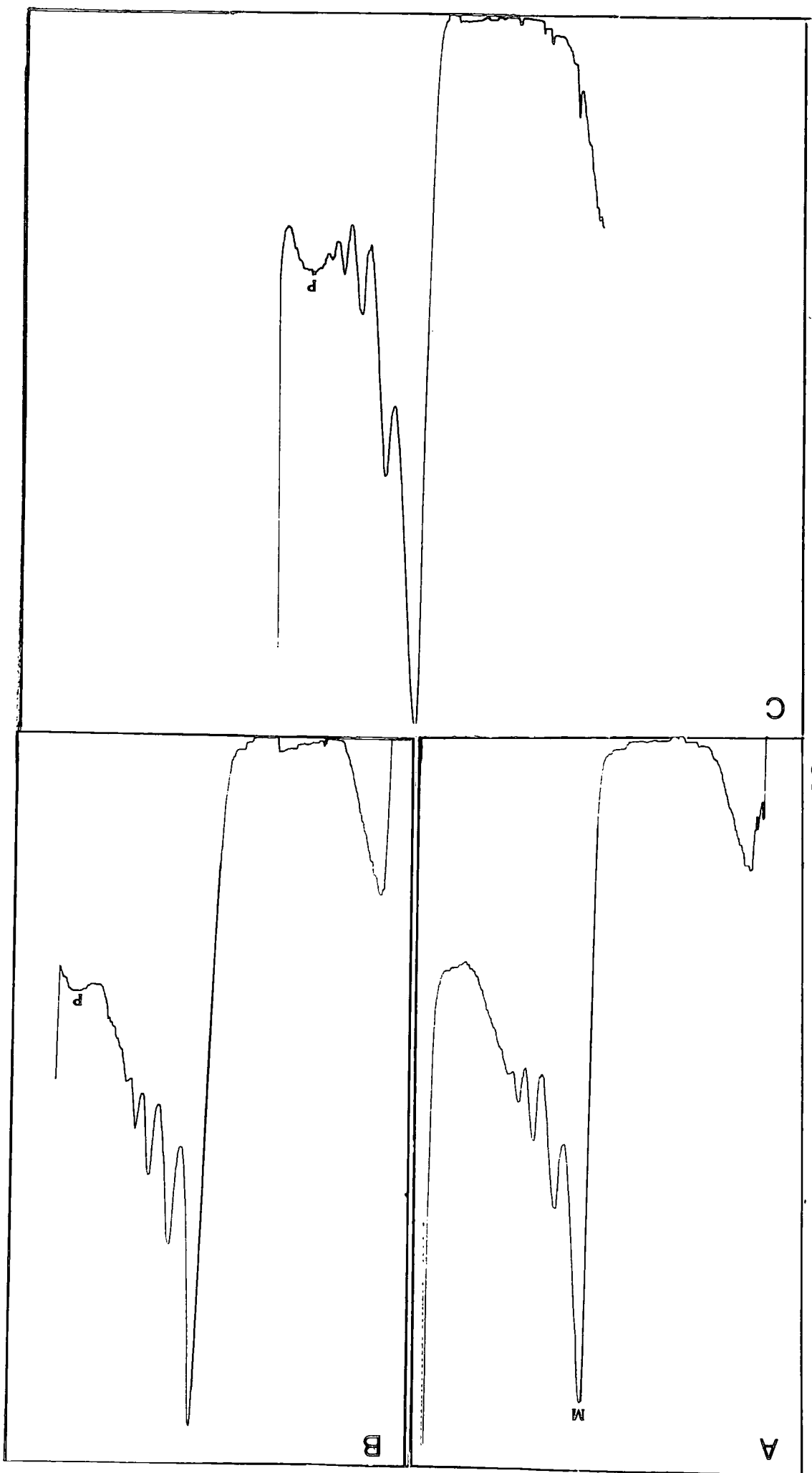
C. 12 hours

M - Monosomes

p - polyribosomes



A<sub>265 nm</sub>



to the breakdown of the subunits of the ribosomes by cycloheximide.

6-methylpurine showed an inhibition of the recovery of the high polymerized polyribosome which aggregated only to the dimer, Figure (72B). Likewise 6-methylpurine showed a similar effect possibly by inhibiting the synthesis of new RNA fractions.

Ribosomes fractions of protoplasts treated with actinomycin D showed a greater level of recovery of polyribosomes, Figure (72A), compared with the cycloheximide and 6-methylpurine treated protoplasts. Thus, a failure to inhibit the regeneration by actinomycin D can be attributed to the ability of polyribosome to recover and produce protein for wall synthesis. Inhibition of the synthesis of the lytic enzyme by cycloheximide (Katz & Rosenberger, 1971; Vries & Wessels, 1975; Grove, 1978; Gooday, 1978) and of RNA required for its synthesis by 6-methylpurine may be the reason for the lack for the regeneration of the protoplasts in these inhibitors. Additionally inhibition of regeneration and wall synthesis by cycloheximide could be attributed to its inhibition effect on the enzyme formation at the ribosomal site. This is also in agreement with the above results.

Figure (72)

PAGE of polyribosomes extracted from protoplasts after 24 hours of regeneration in inhibitors. Culture method was as for (67).

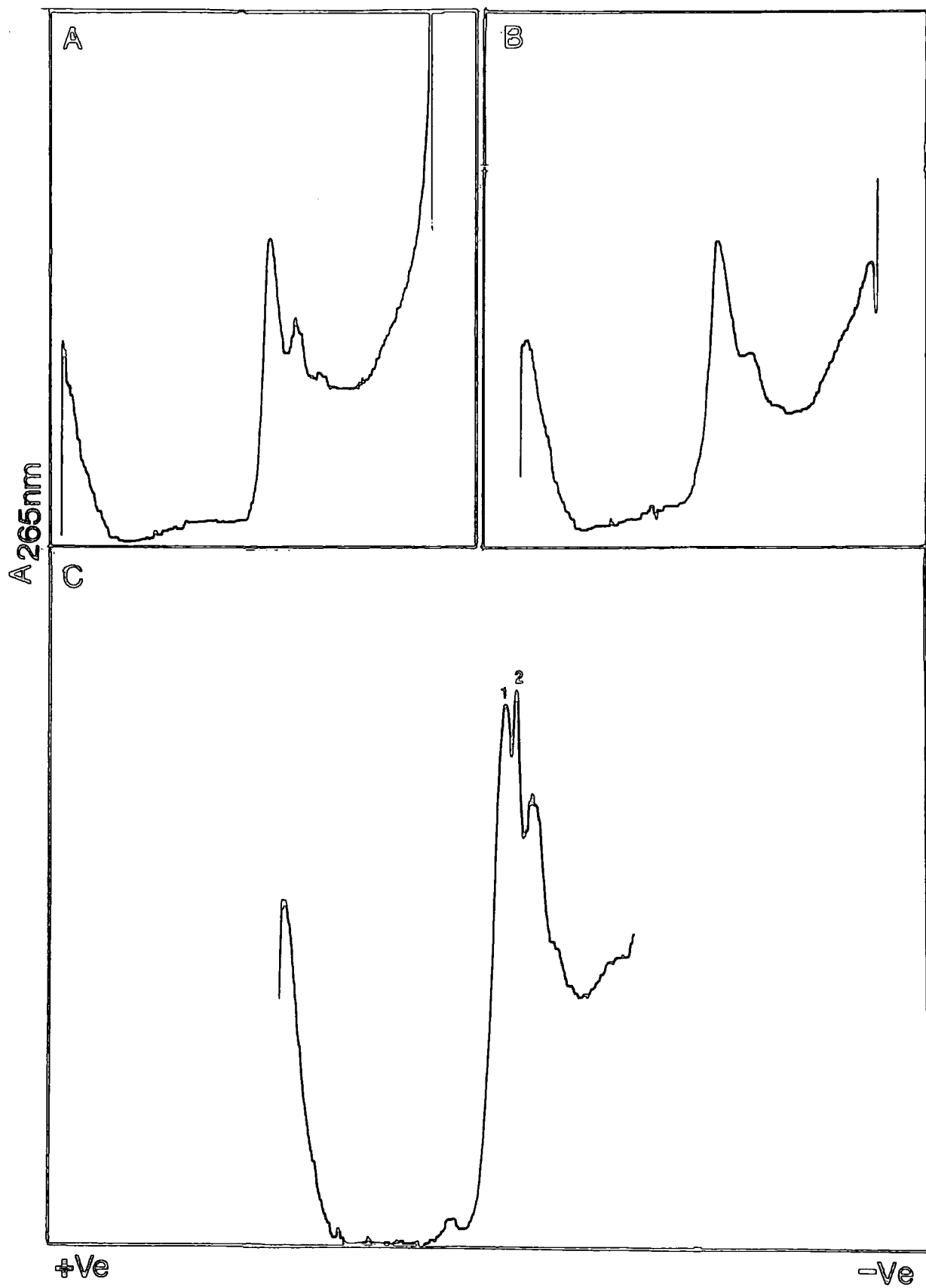
A. Actinomycin D ( $25 \mu\text{g}.\text{ml}^{-1}$ )

B. 6-methylpurine ( $25 \mu\text{g}.\text{ml}^{-1}$ )

C. Cycloheximide ( $20 \mu\text{g}.\text{ml}^{-1}$ )

s - Sub-unit

1 & 2 - Monosomes fractions



30. EFFECT OF HOMOGENIZATION OF THE PROTOPLASTS ON THE LEVELS OF THE POLYRIBOSOMES

As shown in Chapter II page (145), when the protoplasts were homogenized to disrupt the normal distribution of cellular components and also the integrity of membrane, the regeneration appeared to emerge from granular bodies and free from membranous structure. This was interpreted as the precursor or an enzyme of the wall synthesis being intimately associated with granules of a particulate chitin synthase (Ruiz-Herrera et al., 1975; Thomson & Fischer, 1976). So the homogenization may disrupt some cellular components of protoplasts which may carry the wall precursor. Integrity of these organelles or components appears to be required for normal wall synthesis. This was examined in terms of the effect of mechanical disruption on the integrity of ribosomal fraction after homogenization, to investigate if there was any inter-association between wall synthesis and ribosomes integrity.

The stabilized protoplasts which were isolated from stage I sporangiophores were divided into two samples, one subjected to a variety of conditions which mechanically (mortar and pestle or pressure homogenizer) disrupted the protoplasts, then the ribosomes were extracted using the method described in materials and methods. The second

sample was used as control for ribosomes extraction only. Extracted ribosomes were fractionated by 2.2% gels.

Figure (73A) shows that after the disruption the normal integrity of the ribosomes was lost. Breakdown of the ribosomes into their broadening peaks was also seen. In contrast in the control sample, no breakdown of the ribosome was seen and also a series of highly polymerized polyribosomes were observed, Figure (73B).

Breaking the integrity of the polyribosome could be associated with the disruption of the protoplasm. Disruption of the integrity of the pathway of the wall synthesis by the mechanical manipulation may be associated with the breakdown of the ribosomes. Fragmentation of the intact polyribosomes into microsomal units could be in association with the formation of the granular structures which formed hypha. An enzyme could be produced abnormally at microsomal level, since it was found that the microsomal fraction contained chitin synthase (McMurrough & Bartnicki-Garcia, 1971).

The integrity of the protoplasts therefore appears to be associated with the integrity of the polyribosome. During the cutting of the sporangiophore, the abnormal regeneration and branching appeared to be correlated with the degradation of the ribosomes and in affecting the

Figure (73)

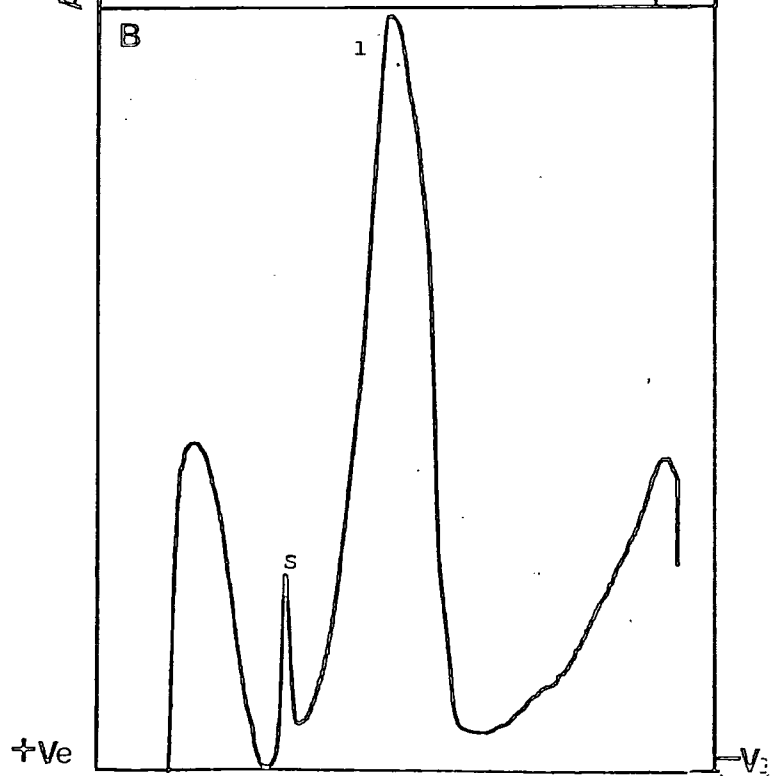
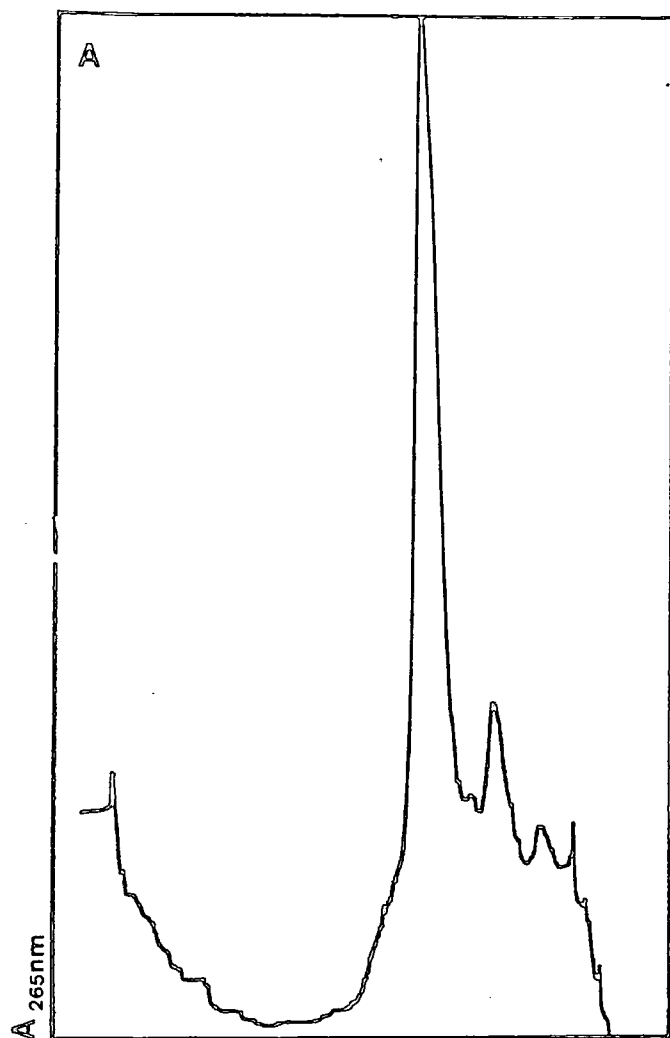
PAGE of 15  $\mu$ g ribosomes, fractionated by 2.2% gels for 2 hours

A. Control

B. After mechanical homogenization

S - Sub-units

1 - Monosomes





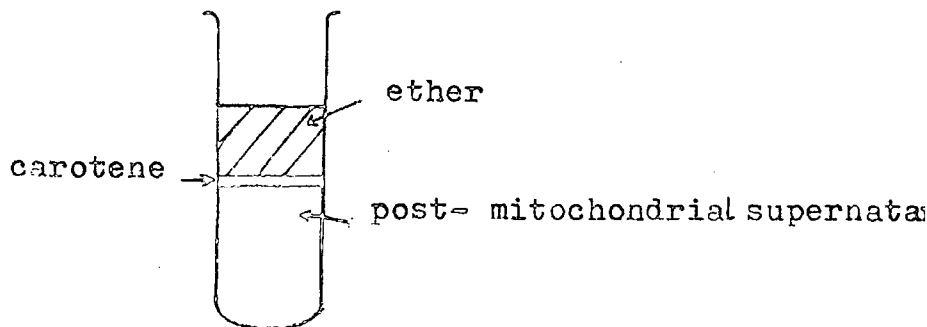
non-uniform distribution of the cytoplasmic organelles and hence the apical growth. Therefore the integrity of the translation level of protein synthesis appeared to be of paramount importance in controlling growth and development of sporangiophores. Presence of more membrane-bound ribosome (Jan, 1974) at the tip of the sporangiophore could be associated with the integral activity at that region. The effect of membrane-bound, and membrane free ribosome on the regeneration was investigated further.

31. ANALYSIS OF MEMBRANE-BOUND AND FREE POLYRIBOSOMES

Jan (1974) found more membrane-bound ribosomes at the tip region than elsewhere in the sporangiophore. In order to establish the pattern of ribosome distribution in this study it was necessary to analyse the sporangiophore for the presence of free or bound-polysomes, in order that any changes during regeneration could be related to the normal growth state.

Two similar methods were used involving detergent; DOC-ionic detergent and Triton X-100, non-ionic detergent. Both digest membranes when added to post mitochondrial supernatant, a problem arises with DOC in that it removes monosomes and possible decreases the total yield of ribosomes as shown for radish leaves (Pearson, 1969).

A normal extraction of ribosomes from the tip, middle and base segments was carried out, but the DOC was replaced by 0.2% Triton X-100 in the grinding medium. In the presence of DOC the post-mitochondria supernatant obtained contained dark yellowish droplets of floating pigments. Such droplets were tested for carotene. 2 ml of ether was added onto a sample of pigment. The tube containing the two separate layers of post-mitochondrial supernatant and ether (see below) was carefully shaken to dissolve the pigment in the ether.



Carotene was determined by measuring the absorption of the mixture of the pigmented ether at a wavelength between (400-500 nm). Figure (74) shows greater absorbance at 450 nm which indicated carotene presence. This indicates that carotene is a membrane-bound component. The post mitochondria supernatant with Triton X-100 or DOC was placed onto the surface of a sucrose solution (see materials and methods Chapter III) and then centrifuged for 3 hours at 40,000 x g using an MSE 65 ultra-centrifuge. Following the procedure for preparing the ribosomal pellet (see above) the u.v. absorptions of the ribosomes of the tip, middle and base segments were measured and using Tester & Dure (1966) equation, the concentration of ribosomes were determined, Table (20).

The total yield of ribosomes were greatly increased in the presence of DOC with the tip segment giving the highest yield. Each value is a mean of 3 replicates, also there was an increase in the yield, in the presence of Triton X-100 detergent compared with the control, but not as great as that found with DOC. Such increases may be attributed to a greater release of the monosomes.

Figure (74)

U.v. absorption spectrum of carotene

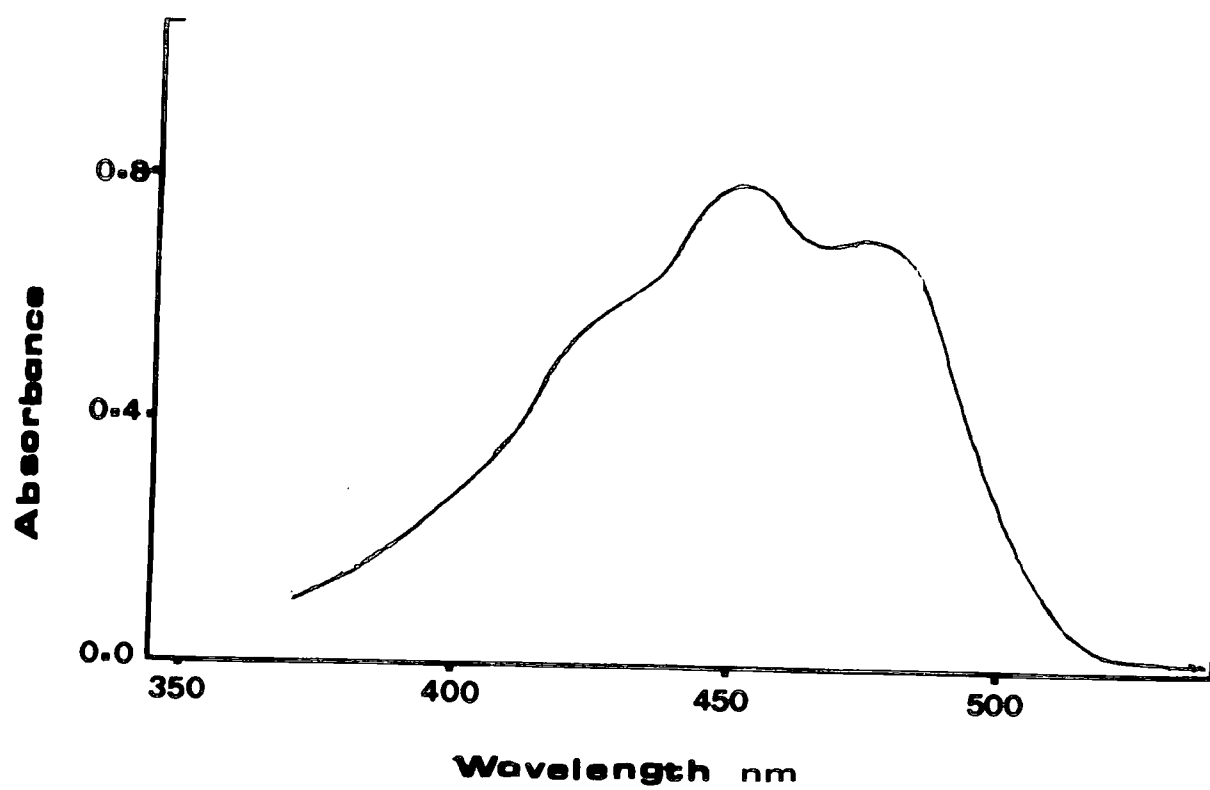


Table (20)

Characteristic of ribosomes extracted in the presence or absence of detergents

|        | Sample   | <u>E<sub>max</sub></u><br><u>E<sub>min</sub></u> | <u>E<sub>max</sub></u><br><u>E<sub>280</sub></u> | Yield $\mu\text{g}.\text{gm}^{-1}$<br>ribosomes, fresh<br>weight |
|--------|----------|--|--|--|
| Tip    | + DOC    | 1.30   | 1.79   | 2050   |
|        | + Triton | 1.20   | 1.34   | 942  |
|        | Control  | 1.10   | 1.60   | 716  |
| Middle | + DOC    | 0.97   | 1.25   | 1382   |
|        | + Triton | 1.06   | 1.12   | 792  |
|        | Control  | 1.18   | 1.29   | 685  |
| Base   | + DOC    | 1.09   | 1.20   | 1145   |
|        | + Triton | 1.06   | 1.15   | 461  |
|        | Control  | 1.18   | 1.4  | 238  |

At the tip, the increased yield of ribosomes was greater than those found for the middle and basal segments. The increase can be interpreted as there being more membrane-bound ribosomes than in other regions, Jan (1974).

### 1. Fractionation of ribosomes

Further analysis of the bound ribosomes in the regions of sporangiophore was made using the polyacrylamide gel electrophoresis system. Scans of polysome fractions from the tip region, Figure (75), showed that DOC increases slightly the level of polyribosomes but had more effect on monosome yield. This could be interpreted as there being more membrane-bound monosomes than free ribosomes. Increased yields in the case of DOC may be due; to the fact that the detergent liberated polysome which, being more fragile, broke down to monosomes.

Middle and base profiles showed no increase in polysome but showed increases in monosome fractions. This could be due to the break down of polysome fractions, since it has been found that there was a polymer present in the control which disappeared when DOC was included.

### 2. Membrane-bound and free ribosomes during the regeneration of sporangiophores

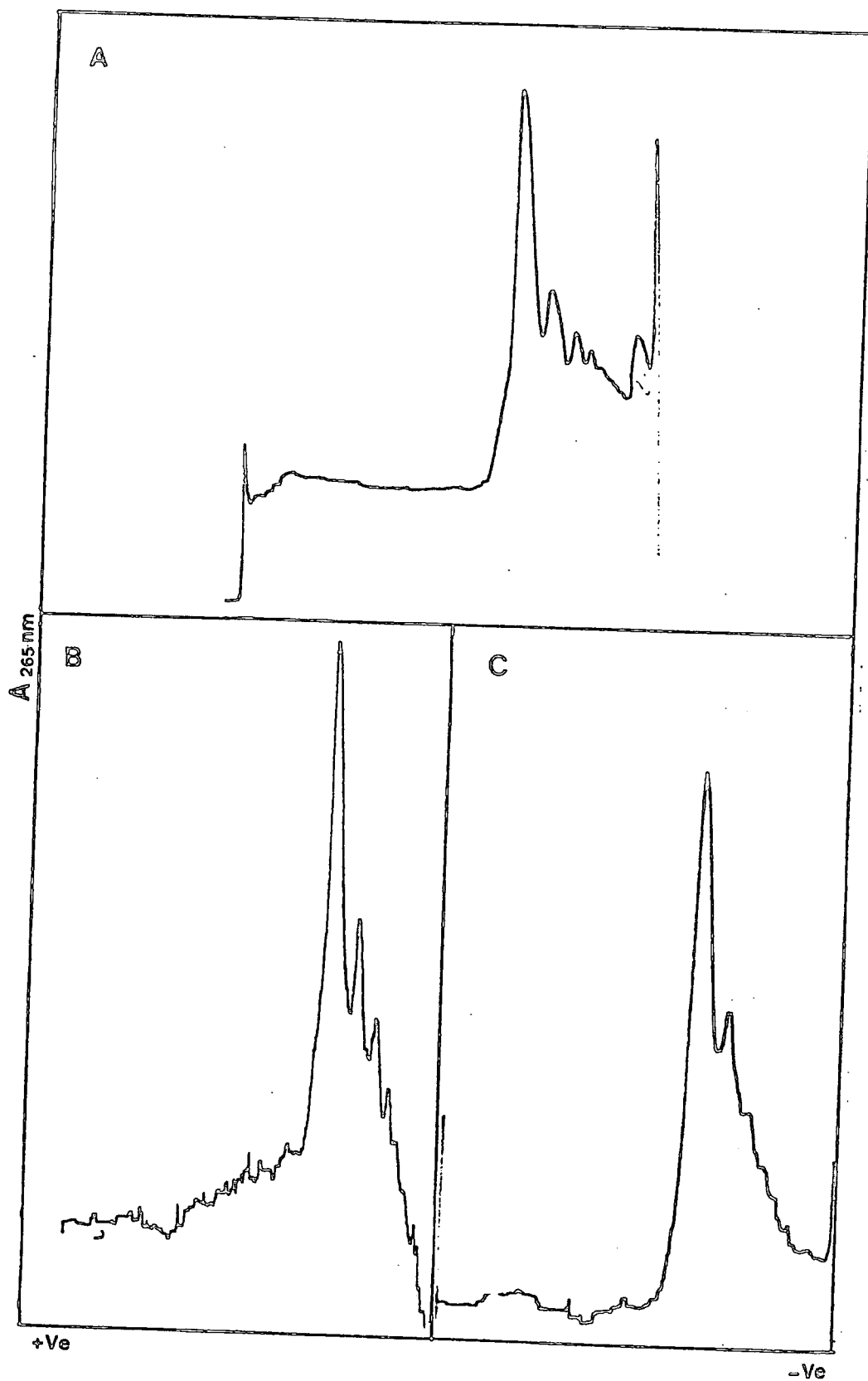
Membrane-bound and free ribosomes were estimated during the regeneration of the tip, middle and basal

Figure (75)

- A. PAGE of total ribosomes extracted from sporangiophores using the normal procedure for extraction
- B. PAGE of membrane-bound and free-ribosomes extracted from sporangiophores with the addition of 0.5% DOC to the post-mitochondrial supernatant. This shows greater level of monomer and dimer ribosomes compared with both A & C
- C. PAGE of membrane-bound and free ribosomes extracted from sporangiophores, with the addition of 0.5% Triton X-100 to the post mitochondrial supernatant

For fractionations, 2.2% gels were loaded with 15  $\mu$ g ribosomes and were run for 2 hours





segments. Times of 0, 4, 8 and 16 hours were chosen for points of extraction and the final yield of bound and free ribosomes were calculated, Table (21).

From the above results, it can be seen that the recovery of the membrane-bound ribosomes was low at the initial stage compared with the membrane-free ribosomes which appeared to be in association with the regeneration. Regeneration time showed a gradation down the sporangiophore, this was reflected in the time for recovery of membrane-free ribosome, this may be related to the metabolic activity at the tip region of sporangiophore.

Membrane-bound ribosomes were seen to increase in yield during a later period especially at the period of completing the growth of stage I and at the initiation of stage II. A greater yield was found at the new regenerated apex of the sporangiophore. This is in agreement with the above observations where more membrane-bound ribosomes were found at the tip of the sporangiophore and also in agreement with the results of Jan (1974). A low recovery of the membrane-bound ribosome during the initial stage of growth of Dictyostelium discoideum was obtained by Bourguignon & Katz (1978). Membrane-bound ribosome appeared to be associated with differentiation for sporulation and production of endogenous mRNA.

Table (21)

Yield of membrane-bound and free from segment of sporangiophore during regeneration

|        | Yield of<br>membrane-bound<br>ribosomes ( $\mu\text{g}\cdot\text{gm}^{-1}$<br>culture) |     |     |     |  | Yield of<br>membrane-free<br>ribosomes ( $\mu\text{g}\cdot\text{gm}^{-1}$<br>culture) |     |      |     |
|--------|--|-----|-----|-----|--|---|-----|------|-----|
|        | 0h   | 4h  | 8h  | 16h |  | 0h  | 4h  | 8h   | 16h |
| Tip    | 206  | 260 | 320 | 896 |  | 634   | 823 | 1 19 | 981 |
| Middle | 112  | 150 | 190 | 452 |  | 381   | 420 | 789  | 886 |
| Base   | .089   | 132 | 171 | 260 |  | 245   | 316 | 587  | 601 |

This mRNA appeared not to be required during the regeneration and growth of the stage I sporangiophore, but it could be utilized during the spore germination (see review by Berry & Berry, 1976).

### 32. GENERAL DISCUSSION AND CONCLUSIONS

The characteristic behaviour of the sporangiophore of Phycomyces during growth and development can be discussed under the following main points; organization and distribution of nuclei, DNA, RNA, ribosomes; the interassociations between the concentration of the cellular components and wall extension, with a special emphasis on the tip region and the possible importance of a balance between wall synthesis and hydrolysis in the growth region. Aspects of morphological growth and development of the sporangiophore which have been noted in the present study, resemble observations reported by Castle (1942, 1959), Gruen (1959, 1967), Gamow & Goodell (1969), Goodell (1971), Bergman et al., (1969), Gruen & Ootaki (1970, 1972), Ootaki & Gruen (1970) and reviewed by Burnett (1976). During growth and development of stage I, nuclei, mitochondria, ribosomes, RNA, DNA, protein and wall polysaccharide were found more concentrated in the tip region. The extreme apex of the sporangiophore has been shown to be devoid of most of the cytoplasmic organelles, however, polysaccharides and proteins are present in both the extreme apex and sub-apical zones.

The overall concentration of organelles and components was seen to decline in the parts below the tip region.

This is in accordance with the observations made in fine and ultrastructural studies (Zalokar, 1969; Peat & Banbury, 1967; Marchant et al., 1967; Thornton, 1968b; Bergman et al., 1969; Tu et al., 1971; Tu & Malhotra, 1976; Trinci, 1978b) which have shown the presence of nuclei, ribosomes, and endomembrane at the sub-apical zone, while the extreme apex has been shown to contain numerous secretory vesicles. A greater concentration of polysaccharides and protein at the apex was thought to be in association with vesicles, since it has been found that vesicles may carry the wall substances such as polysaccharides or discharge enzymes (Girbardt, 1969; Grove, 1970; Trinci, 1971, 1974; Bartnicki-Garcia, 1973; Rosenberger, 1978 and reviewed by Weber & Hess, 1976).

Results described in this work on extraction and fractionation of RNA species and ribosomes have shown a greater content of polyribosomes 28S, 18S RNA (high molecular weight) and also 5S and 4S RNA (low molecular weight) at the tip region than at the middle and basal region of sporangiophore. In addition, more obviously 5.8S RNA has been found free at the tip in contrast to other parts of the sporangiophore. A greater content of 5.8S and 5S RNA has been found to be important in the biosynthetic activity of ribosomes of Saccharomyces cerevisiae (Helser & McLaughlin, 1975) and also it has

been reported that a unique synthesis of 5.8S has been attributed to the higher activity of the cell in producing protein. Recovery of high levels of polyribosomes and free 5.8S RNA was also found during the maturation and growth of Phycomyces mycelium (Pearson, unpublished work). The presence of highly polymerized polyribosome during growth of fungi (Berry & Berry, 1976) and in other living systems, (Pearson, 1969; Spirin & Gavrilova, 1969; Ramakrishnan & Staples, 1970; Strangeway, 1977) has been shown to be associated with formation of active mRNA and thus active metabolic activity. A greater content of membrane-bound ribosomes, which has been found at the tip region of the sporangio-phore, resembles the results reported by Jan (1974) for Phycomyces. He also found a higher concentration of chitin synthase at that region, and attributed this to active wall synthesis. The recovery of high levels of membrane-bound ribosomes during growth of some filamentous fungi (Hollomon, 1971; Mirkes, 1974; Bourguignon & Katz, 1978) was attributed to the formation of a unique differentiation during growth of these fungi. The rate of synthesis of RNA and polysomes (thus mRNA) regulates the synthesis of enzymes and also plays a role in determining fungal growth (Killick & Wright, 1974 and reviewed by Bramble et al., 1978 and Smith, 1978).

A greater content of tRNA (4S) at the tip region of the sporangiophore could also be attributed to more protein synthesis and an active translational level. This is in agreement with an observation reported for Neurospora crassa by Vanderhoff et al., (1972). Additionally, and as yet not fully characterized RNA species, 20S, was found in the sporangiophore. The presence of this appeared in agreement with the observations that a similar new RNA fraction appeared during sporulation of Saccharomyces cerevisiae (Udem & Warner, 1972; Hopper et al., 1974; Weijksnora & Höber, 1978).

The vacuolation zone has been shown to be initiated at a region below the sub-apical region of the sporangiophore. The size of the vacuole has been found to increase with distance from the apex. Increase in the size of the vacuole has been shown to be associated with a declining concentration of cellular components. Increased numbers of vacuolated protoplasts at parts below the tip region also were used as evidence for the stability of vacuolation.

It was concluded that the non-uniform distribution of cellular components and growth along the sporangiophore, may be indicative symptoms of ageing. Apart from the base, growing sporangiophore of Phycomyces can be



separated into at least three regions, viz. (i) an extreme apex region with higher concentration of wall precursors and protein, (ii) a sub-apical region rich in ribosomes, nuclei, RNA, DNA, mitochondria and endoplasmic reticulum, (iii) a vacuolation zone. A similar zonation was described for the hyphae of filamentous fungi (reviewed by Fenc1, 1978) and was attributed to the age of the compartments (see also review by Grove, 1978). Whether the distribution of the organelles and components was authentic and thus reflected a significant physiological state of the apical growth of the organism, or whether they just arose as mere artifacts during processing needs discussion.

The distribution of organelles and components especially nuclei, mitochondria, RNA, DNA and protein along with the sporangiophore were determined cytologically using different staining methods which gave similar results as seen by Peacock (1958), Gurr (1960), Jensen (1962), Cohn (1969). Techniques and precautions for maintaining normal distribution and an intact tip as applied to studies of hyphae were also taken into account in this study. The techniques and precautions were extensively described by Ward (1888); Burgeff (1915), Smith (1923), Castle (1958), Robertson (1965, 1968), Bracker (1967), Marchant & Smith (1968),

Grove et al., (1970), Katz & Rosenberger (1970, 1971), Gooday (1970), Bartnicki-Garcia (1973), Grove (1978). Uniform fixation and staining were obtained by using sporangiophores which had been treated to remove the cuticle layer. This minimized difficulties faced with regard to the penetration of fixatives and stains and therefore gave preparations without disorganization and dislocation of cytoplasm. Procedures used for fixation and staining for cytoplasmic organelles and components of sporangiophores were similar to those used and described by Hepden & Hawker (1961), Abbot (1964), Hawker et al., (1968), Davison (1968a,b), Flanagan (1970), Frank & Reau (1971), Myers & Cantino (1971, 1974), Wong & Gruen (1977), Hutter et al., (1978).

Within the tip region, RNA, DNA, protein, nuclei and mitochondria are much denser at the sub-apex region of the sporangiophore. A greater density of staining of RNA, DNA, protein, nuclei and mitochondria was also found at the same region of the sporangiophore of Peronospora parasitica (Davison, 1968a,b). Wong & Gruen (1977) have reported that the growing zone of the fruit bodies of Flammulina velutipes is different from other regions, in that it contains more cytoplasm, and they reported that this may be attributed to metabolic activity. A great accumulation of RNA and

protein during the growth of Allomyces sp. Blastocladiella emersonii and Neurospora crassa was found to be in association with the morphogenesis of the fungi (Turian & Viswanathan, 1966; Myers & Cantino, 1971, 1974; Lovett, 1975; Turian, 1975; Bramble et al., 1978).

The gradient of macromolecules and organelles appear to be under the influence of gene controlled polar growth (see review by Fencel 1978). Uniform distribution of organelles was also attributed to the polarity assembly of organelles and apical growth (Robertson, 1965, 1968 and Grove, 1978). The apical growth pattern has been found to be maintained by non-uniform distribution of cytoplasm of Pythium, Phytophthora, Dictyorus, Saprolegnia, Blastocladiella emersonii, Neurospora crassa, Schizophyllum commune, and Polystictus versicolor and other filamentous fungi (Hemmes & Hohl, 1971; Grove et al., 1970; Barslow & Lovett, 1969, 1974; Heath et al., 1970; Hunsley, 1973 and reviewed by Burnett, 1976). Furthermore it has been shown that the accumulation of organelles and components at the tip region is independent of the cytoplasmic streaming, since it has been found that the cytoplasmic streaming from the tip to the basal regions is more than the movement from the base to the tip regions (see Bergman et al., 1969; Jennings, 1976). The translocations of

the nutrients in the fungi has been found to be independent of the cytoplasmic streaming (Howard, 1978). It has been concluded that new protoplasm is formed throughout the hyphae and transported to the tip of filamentous fungi by active streaming (Zalokar, 1959b). In contrast, Fencel (1978) disputed these observations and regarded nutrient transport and protoplasmic streaming as being dependent on the cytoplasmic streaming, therefore the association between the two processes is under some doubt.

In this study isolated sporangiophores have been shown to reach the same stage as do attached sporangiophores. It also appears that the isolated sporangiophores may depend on the preformed substances during the growth of stage I. Growth and development of isolated sporangiophores appears as a system of local autonomy or as an endotrophic response, (Gamow & Goodell, 1969). Observations in this study are also in agreement with the results observed by Gruen (1959), Bergman et al., (1969). The final stage of development (IV) has been shown to be achieved even with fragments or segments of sporangiophores. The tip segments has been shown to be the more active region during regeneration and development. Similar observations were also reported by Gruen & Ootaki (1970, 1972) , Ootaki & Gruen (1970). Furthermore the protoplasts which were

isolated from the tip region have also been shown to produce a greater quantity of wall substances and to develop a hyphae in a much shorter time. The recovery of RNA, ribosomes and protein was found to be greater during the regeneration of both tip segments and their protoplasts, compared with those of the middle and base segments and their corresponding protoplasts. These results indicate evidence that the tip region shows more activity during growth and development, possibly because it is able to produce more protein. This is probably due to its greater content of RNA, DNA, nuclei and ribosomes. The elongation of regenerated sporangiophores has been shown not to require a connection with the substrate hyphae or exogenous water or nutrients. The possibility is that assumed growth can be established at the expense of neighbouring compartments (Gooday, 1974).

Degradation of cytoplasmic organelles and components in the basal region and the maintenance of normal concentrations of cytoplasmic components at the tip region has been shown during the isolation and regeneration studies. The degradation of cytoplasmic contents takes place during starvation situations during fungal hyphae growth reviewed by Fencel (1978). It has been reported that the main cause of degradation or autolysis of

organelles appears to be the imbalanced condition along the hypha. The imbalanced condition has been found to be induced by either internal or external factors; disturbance of the organelles, mechanical activation of some enzymes which influenced or disturbed the intracellular structure of the cell wall, the lack of nutrient (starvation) as a general energy source or by a specific nutrient deficiency of some substances due to its localisation (ageing) along the hyphae (see review by Fenc1, 1978). Degradation or autolysis of the older organelles or components at the basal region, after sporangiophore isolation, can also be considered as an internal adaptation system (compartmentlization) throughout the sporangiophore. By this means the older contents can be degraded and utilized again by the younger region (tip) of the sporangiophore. Degradation products of RNA, ribonucleoside monophosphate, release during starvation of Bdellovibrio bacteriovorus have been found to be utilized and used as biosynthetic precursors for synthesis of both nucleic acid and other cell material, and additionally the ribose sugar has been shown to be used as a source of energy (Hespall & Mertens, 1978).

Degradation of RNA at the basal region during the isolation (imbalanced condition) and alternatively the

maintained RNA concentration at the tip region was used as evidence for the reorganization or redistribution of cytoplasm during regeneration and growth of sporangiophore. Thus the ability of the tip region to maintain the apical growth extension may be attributed to its being a younger region receiving a continuous supplement of degraded components. This is due possibly to its unique content of cellular components, since Trinci & Righelato (1970) stated that autolysis does not proceed synchronously in the entire filament but only in its individual components. They also found that the decomposition of the organelles of the same type is synchronous (i.e. it is catalysed by cell free enzyme). So the lysosome and autophagy do not play a substantial role in the autolysis; also vesicles with lysosomal function (Thornton, 1968a; Burnett, 1976) are rather associated with lysis and resynthesis of cell wall (Mahadevan & Mahodkar, 1970). Trinci (1974) assumed a continuous supply of the enzyme containing vesicles, including the wall building blocks from the older regions toward the apical parts of the hyphae. This, because of the unique non-uniform distribution of organelles associated with the apical growth, possibly gives that region a special autolysis resistance. Degradation of cytoplasmic organelles at the basal region of sporangiophores during growth is in agreement with the observations reported by

Nagazaki (1968b), Righelato et al., (1968), Trinci & Righelato (1970), and Fencel (1978), since it has been found that the degradation of cytoplasmic organelles were in association with the degradation of ribosomes.

Regeneration and branching at the tip region after mechanical cutting could be attributed to the imbalanced condition brought about in the cell by disturbance of the uniform distribution of organelles. Thus loss of the balanced condition (Trinci, 1978b) between the lysis and deposition of wall substances thus maintained the apical growth but in an abnormal fashion.

Degradation of RNA and polyribosomes and other cytoplasmic components at the basal region (stage II) of the sporangiophore could also be attributed to the starvation (Bergman et al., 1969). Growth of stage IV thus may depend on the autolysed substances which were originally supplied by the mycelium during the growth of stage I. Increased contents of tRNA (4S) at the intercalary region during the elongation growth of stage IV could result in an increase in the aminoacyl-tRNA and thus protein synthesis. Bull & Bushell (1976) noted that phototropic response of fungi in general involves an increase in acolation tRNA and similar processes may occur in the sporangiophore. Light was also found to increase the content of RNA



(Tan, 1978), during the phototropic response. Thus degradation of cytoplasmic contents of the basal region of stage IV is compensated for by an increase RNA species in the intercalary region where more protein synthesis is required for the production of wall substances. The decline in recovery of polysomes in the autolysed region of the sporangiophore could be related to the reduced recovery of free 5.8S RNA, since less free 5.8S has been found in older hyphal preparations (Pearson, personal communication). In this study it has been found that free 5.8S was always associated with apical growth of sporangiophores. Thus sporangiophore ageing resulted in a lack of an equivalence or compartmentalization region along the sporangiophore and was reflected in RNA and polysome changes. Likewise direct continuity between sporangiophore apical growth and the synthesis of new wall necessitates a localized requirement and recovery of RNA and polyribosomes.

The essential requirements for the recovery of RNA, polyribosomes and protein during growth and development of the sporangiophore were tested by incubating the sporangiophore and also protoplasts of sporangiophores in nucleic acid and protein synthesis inhibitors. Different effective inhibitors were used (see page 102). Cycloheximide (as an inhibitor of protein synthesis, (Fritz & Ischesche, 1971;

Hadholov & Nikolaev, 1976; Grove, 1978) and 6-methyl-purine analogue (RNA synthesis inhibitor Kersten & Kersten, 1974 and see page 100 ) have been shown to inhibit the growth and development of both sporangiophore and protoplast. Cycloheximide has also been shown to inhibit the germination and growth of Phycomyces spores and mycelium (Van Laere et al., 1976, 1977). Van Leare et al., (1976, 1977) have attributed the effect of the cycloheximide to its ability to inhibit the synthesis of enzymes of wall synthesis at the translation level. Likewise the recovery of polyribosomes was shown to be impaired by the cycloheximide. In addition 6-methylpurine was found to impair the recovery of total RNA.

The lack of an effect of actinomycin D on the growth and development of sporangiophore suggested that sporangiophore growth is not dependent on new mRNA synthesis but appears to be dependent on preformed mRNA which may be present at the site of translation level. These similar effects and suggestions are in agreement with similar results reported by Mayo et al., (1968), Hadholov & Nikolaev (1976), Youatt (1976), Cohen (1977), Ernst & Leinick (1977), Fisher (1977), Ramadani (1978) and also reviewed in fungi in general by Fincham et al., (1979). Thus the continuous growth of the sporangiophore appeared

to be dependent on the presence of RNA and polyribosomes and thus ensuring continuous synthesis of protein. Inhibition of continuous synthesis of wall lysis enzymes and also chitin synthase was related to the effect of cycloheximide during growth and regeneration of Aspergillus niger hyphae and protoplasts of Schizophyllum commune (Vries & Wessels, 1975 and see review by Gooday, 1978).

The production of specific proteins or enzymes along the hyphae of fungi has been reported to take place at different loci, and thus suggested to be dependence on the age of both the hyphae and organelles (Zalokar, 1959b; Yanagita, 1966; Yanagita & Nomachi, 1967; Nagazaki, 1968a,b; Fenc1, 1978). It was also suggested that the distance between the site of the synthesis and the action of the enzymes determines the physiological state of fungal hyphae. The regeneration and growth extension are active metabolic processes and this is related to the activity of chitin synthesis in terms of synthesizing new wall material via a re-distribution or re-organizing or pre-existed cell material. As part of the hyphae, the cell wall may also undergo an ageing process. Marchant & Smith (1968), Strunk (1963), Gull & Trinci (1974) have found structural differences between the wall of the terminal growth apex

and that of the lower part of the hyphae. The correlation between the polar growth of the sporangiophore and the biosynthesis of wall (see Chapter I) resembles that discussed by Bartnicki-Garcia (1973), Gooday (1978), Trinci (1978b), Katz & Rosenberger (1971), Gooday (1971) and reviewed by Burnett (1976). They showed that although wall elongation is limited to the apical part of the growing hyphae, thickening of the wall may take place even in parts more distant from the apex. In this study lysis of the wall was found at the apex region of the fungal sporangiophore during growth extension (see Chapter II). More lysis at the tip was also observed during protoplast isolation, and this may indicate that the apex region is more able to lyse than other region along the sporangiophores, since it has been found that older regions of the sporangiophore contain more melanin and sporopollenin as protective substances (Furch & Gooday, 1978). Such resistance could also contribute a useful system in maintaining polar growth. Additionally, maintenance of apical growth extension of sporangiophores has been illustrated to be achieved in terms of a balance between lysis of the extreme apex region and at the same time deposition of chitin at the sub-apical region (Trinici, 1978b). Therefore it can be concluded that the

growth and development of the sporangiophore is led and regulated by the extreme apex (1-2 mm).

The activity at the apex region appeared to be correlated with the activity of the sub-apical region where the continuous supply of wall precursors are controlled. The interassociation between the extreme apex and sub-apical regions in achieving a balanced growth needs further discussion related to other alternative systems.

Fungi are believed to transport their hydrolytic enzymes enclosed in vesicles toward the apex of hyphae where growth extension takes place (Girbardt, 1969). Vesicles may also produce a material for cell wall formation (Grove et al., 1970) and see review by Bartnicki-Garcia (1973). A continuous supply of the enzyme containing vesicles including wall building blocks, was also assumed by Trinci (1971, 1974). The formation of vesicles from endomembrane systems has been reviewed by (Weber & Hess 1976). The essential requirement of nuclei and polyribosomes in synthesis vesicles was also investigated (see Bartnicki-Garcia, 1973; Weber & Hess, 1976), and it has been shown that actively growing hyphal apices accumulates numerous vesicles (Brenner & Carroll, 1968; McClure et al., 1968; Hemmes & Hohl, 1971; Grove & Bracker, 1970). A similar accumulation of vesicle was found as a

conglomeration in yeast (Cortat et al., 1973; McCully & Bracker, 1972; Holley & Kidby, 1973). Numerous vesicles were also found at the extreme apex of sporangiophore and have been observed to coalesce with the plasmalemma of sporangiophore (Peat & Banbury, 1967; Marchant et al., 1967; Thornton, 1968b; Tu et al., 1971; Tu & Malhotra, 1976).

It was suggested by this study that the activity of the sub-apical region of the sporangiophore is concerned with the formation of precursors of wall substance synthesis, since it has been characterized in containing more endoplasmic reticulum, and more total ribosomes (Jan, 1974). Likewise it was suggested by Trinci (1978b) that the migration of vesicles through the extreme apex may be regulated remotely from the sub-apical region. Nuclei may also regulate the synthesis of vesicles (Weber & Hess, 1976). Additionally more intensity of stain which determined polysaccharide and protein at both the regions of sub apex and extreme apex of the sporangiophore was used as evidence in the present study that the vesicle may contain both the lysis enzyme and wall microfibril. Polysaccharides as a wall substrate have been found to be carried by vesicles (see review by Rosenberger, 1976).

As described in Chapter III an interrelationship between ribosomes and the production of wall microfibrils has been found during regeneration studies since a maximal recovery of polyribosomes has been shown to be in association with initial synthesis of the wall substances. Likewise microsomal fractions of Mucor rouxii were found to stimulate the synthesis of chitin synthase (McMurrough & Bartnicki-Garcia, 1971). Additionally a greater content of chitin synthase was found at the tip region of the Phycomyces hyphae (Ruiz-Herrera & Bartnicki-Garcia, 1976b). The pathway of synthesis of chitin synthase of Phycomyces blakesleeianus and Mucor rouxii was found to be similar (McMurrough & Bartnicki-Garcia, 1973; Ruiz-Herrera & Bartnicki-Garcia, 1976a, see also Thomson & Fischer, 1976; Fischer & Thomson, 1979), and also has been found to be similar to that of yeast.

It is important to suggest that chitin synthase may be carried by vesicles in an inactive form which can be activated at the plasmalemma, during the lysis process at that region of extreme apex. But it can be activated in vitro by homogenization (as shown in the protoplast experiment) or by cutting the tip region, where lysis enzyme is released as a result of damage which then activates the chitin synthase and thus produces abnormal

growth. Similarly it was found (McMurrrough et al., 1971; Ruiz-Herrera et al., 1975) that chitin synthase was carried by a granular like structure in Mucor rouxii. Sentandreu & Ruiz-Herrera (1978), reported that most of the wall enzyme is located inside the permeable barrier of the cell in the form of an inactive precursor; the enzyme and its activating factor (protease?) were reported to be in separate locations within separate compartment of the cell. Thus chitin synthesis and wall lysis could be regulated by the rate of flow of vesicles (Stewart & Rogers, 1978) from the polysaccharide polypeptide synthesizing and assembling organelles to the growing points of hyphal cell wall.

Ribosomes are not usually present in the extreme apex of the sporangiophore where an endoplasmic reticulum has been found of limited dispersion. Thus it is suggested that any ribosomal activity, i.e. genesis of polypeptides and protein, must occur at regions where more ribosomes, RNA, nuclei, are present (sub-apical region). In this case the synthesis and migration of substances and vesicles may be regulated remotely by the sub-apical regions of the sporangiophore. The granular nature of vesicles at the extreme apex has been found to be similar to that of the region below the apex and both were regarded as smooth surfaced, i.e. ribosome free (see Peat & Banbury, 1967;



Marchant et al., 1967 and general reviews by Grove, 1978). When membranes of two or more vesicles appear joined, two or more inclusion are evidentially suggested in the common lumen (Weber & Hess, 1976). Thus intense polysaccharide and protein staining in the apex region may indicate that the vesicles may carry the discharged latent enzyme, and also may increase the plasmalemma.

The apical vesicles have been found to have a membrane similar to that of the plasmalemma but unlike the membrane of nuclei and E.R. . If this is to be interpreted as a continuous endomembrane system there must be transition during membrane displacement from nuclear envelope to the vesicles (Bartnicki-Garcia, 1973). The results in this study (Chapters I & II ) which showed more polyribosome recovery and abundance of envaginated nuclei at the time of wall synthesis, were taken as evidence of the presence of the interrelationship between cell wall substances synthesis and nucleic acid and protein synthesis. Also the particles which produced chitin synthase may be carried by granular vesicles and these particles may be proteinaceous. Otherwise vesicles may be involved in secretion, possibly, of enzymes.

The presence of more ribosomes in the sub-apical region (Jan, 1974) and also a greater content of poly-

ribosome seen in the present study may have contributed to the presence of double membrane vesicles at the sub-apical region of the sporangiophore. This was suggested when Steel (1973) found the double membrane abundant with polyribosome and suggested that it may represent the site of synthesis of wall enzymes of the fungus Geotrichum candidum. The endomembrane apparatus is not believed to take part in protein synthesis and it appears unlikely that in growing hyphae the tapered tip is involved with membrane protein synthesis, due to the infrequent presence of ribosomal units or rough endoplasmic reticulum. Ribosomes are found in greatest abundance in the sub-apical, non-vacuolated zone wherein there is a consistent association between endoplasmic reticulum and endomembranes.

Thus it can be concluded that the growth and development of sporangiophore depend on the sub-apical region, where greatest content of polyribosomes, nuclei, RNA and membrane are found. These are the important organelles and components in synthesizing wall precursors. Cytoplasmic vesicles (cavity wall precursors) also play a major part in the growth of the sporangiophore by which essential new membranes for the plasmalemma are supplied, while also transporting the essential components for new wall

synthesis. The vesicular hypothesis for carrying a wall enzyme(s) or substances for cell extension has been almost totally concluded from the protoplasts and regeneration studies and it is only recently that attempts have been made to hypothesize regarding the condition of regeneration that occur during the manipulation (Ruiz-Herrera et al., (1975) . Additionally general growth and development of the sporangiophore of Phycomyces has been shown to be regulated by the tip (2-4mm) region. It appeared to embody mechanisms for controlling the unique differentiation, assembly of wall elements and modulation of timing of morphogenesis, and this appeared to be autonomous. Apical growth of the sporangiophore can be considered as the result of integrated reactions of the cytoplasmic organelles and compounds under a balanced situation. The observations which were obtained by the present study, support a model for hyphal tip growth which highlights a special function of nuclei, RNA and ribosomal components.

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APPENDIX

1. Fixatives

1. Acetic acid - Alcohol

|                      |       |
|----------------------|-------|
| Glacial acetic acid  | 5 ml  |
| Ethyl alcohol (100%) | 15 ml |

2. Formalin - Acetic acid - Alcohol

|   |       |
|---|-------|
| Commercial formalin<br>(40% formaldehyde) | 10 ml |
| Glacial acetic acid                       | 15 ml |
| Ethyl alcohol (50%)                       | 5 ml  |

3. Formalin - Acetic acid

|                     |       |
|---------------------|-------|
| Commercial formalin | 10 ml |
| Ethyl alcohol (50%) | 5 ml  |

4. Formalin - Alcohol

|                     |       |
|---------------------|-------|
| Commercial formalin | 10 ml |
| Ethyl alcohol (95%) | 15 ml |

5. Glutaraldehyde

|                                       |       |
|---------------------------------------|-------|
| Glutaraldehyde                        | 10 ml |
| 0.1 M Sodium phosphate<br>buffer pH 7 | 10 ml |
| Distilled water                       | 80 ml |

6. 4% Glutaraldehyde

|                                       |       |
|---------------------------------------|-------|
| Glutaraldehyde                        | 40 ml |
| 0.1 M Sodium phosphate<br>buffer pH 7 | 40 ml |
| Distilled water                       | 20 ml |

2. Preparation of Egg Albumin

1 gm Sodium salicylate

50 ml White of egg

50 ml Glycerine

Dissolve sodium salicylate in water, add the  
rest, mix and filtrate

3. Preparation of Stains

1. Fuelgen stain

5 gm Basic fuchsin

100 l N hydrochloric acid

5 gm Sodium metabisulphite

2.5 gm Activated charcoal

Mix the fuchsin in 1000 ml boiling water and  
cool to 50°C. Add the hydrochloric acid and  
sodium metabisulphite and shake for 10  
minutes. Add the charcoal and store in the  
dark for 24 hours, the filtrate was kept in  
the dark until needed.

2. Pyronin y

0.1 gm Pyronin y was dissolved in 100 ml  
distilled water

3. Toluidine blue

0.1 mg Toluidine blue was dissolved in  
100 ml distilled water

4. Methyl green

0.15 mg crystallized methyl green was  
dissolved in 100 ml distilled water

5. Acridine orange

0.1 mg Acridine orange was dissolved in  
100 mg of 0.1 M phosphate buffer pH 7

6. Giemsa stain

Stock solution of Giemsa stain was supplied  
from BDH. 1 ml of stock solution of stain  
was diluted with 10 ml of 0.1 M phosphate  
buffer pH 7.

4. Preparation of Sulphur Dioxide Water

50 ml Sodium metabisulphite (10% solution)  
+  
50 ml 1 N hydrochloric acid  
+  
Distilled water to 1000 ml



## 5. Chemicals

All materials used for staining were of BDH grade. Materials used for growth media for extraction and estimation Macromolecules were of Analar grade.

